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MASTERARBEIT

Titel der Masterarbeit

„Generation and characterisation of selected mutants of the cyanobacteria *Nostoc* sp. PCC 7120 and *Synechocystis* sp. PCC 6803“

verfasst von

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Abstract

Cyanobacteria are the only bacteria that are capable of performing oxidative photosynthesis. These bacteria are among the oldest living beings on the planet and shaped todays atmosphere. They were the first organisms that used water as reduction equivalent and were among the first that developed respiration. Therefore they are often used as model organisms.

Photosynthesis and respiration share components of the electron transfer chain. In low light or darkness respiratory terminal oxidases (RTOs) are used to produce energy by reducing O_2 to water. Three different types of RTOs are known in cyanobacteria, heme copper oxidases, quinol oxidases and plastidic terminal oxidases. The group of heme copper oxidases contains the mitochondrial type cytochrome c oxidase (Cox), which is the most important oxidase for respiration in cyanobacteria. But this group also includes the alternate respiratory terminal oxidases (ARTO). These are homologues to the Cox with slight difference in the active sites of the subunits I and/or II. The ARTO type II is also thought to have a Cox activity but this has never been shown before.

A vector was constructed for the knock out of the ARTO type II in *Nostoc* sp. PCC 7120. For further investigations of the RTOs and the heterotrophic growth of this strain on fructose, mutants with different combinations of knocked out RTOs were generated by conjugation. The characterisation of these mutants is yet to be made.

All cyanobacteria can grow photo-litho-autotrophically and some are capable of other growth modes too, when suitable organic molecules as energy, electron and/or carbon sources are available. The cyanobacterium *Synechocystis* sp. PCC 6803 can use glucose for heterotrophic growth but is sensitive to fructose. It has been shown in this work that a fructose resistant mutant of this strain can grow heterotrophically on high amounts of fructose with a dramatic increase of the growth rate. The fructose may enter the bacterium via a so far unknown sugar transporter. Therefore two possible sugar transporters – *lacF* and *melB* – were knocked out. The destruction of *lacF* led to an even more enhanced heterotrophic growth rate. It was shown that more fructose was incorporated in cultures that were pretreated with fructose. The gene product of *lacF* may have a regulatory function regarding the uptake of fructose.

The *melB*$^-$ mutants did not become homozygous within 12 weeks, which indicates that this gene is essential to the organism. However, its function remains unclear.

Introduction

About Cyanobacteria

As old as 3.5 billion years, cyanobacteria are among the oldest organisms on the planet (Schopf, 1993). Cyanobacteria are prokaryotic organisms that can perform oxygenic photosynthesis and use light as their primary source of energy (Stanier, 1973). It is likely that these organisms first developed aerobic respiration (Broda, 1975). Some cyanobacterial strains can fix nitrogen as well (Vermaas, 2001).

Cyanobacteria can live under very different environmental conditions all over the world. According to the endosymbiotic theory chloroplasts of higher plants and cyanobacteria share the same ancestors (Mereschkowski, 1905). As the first organisms that produced molecular oxygen, cyanobacteria shaped today's atmosphere.

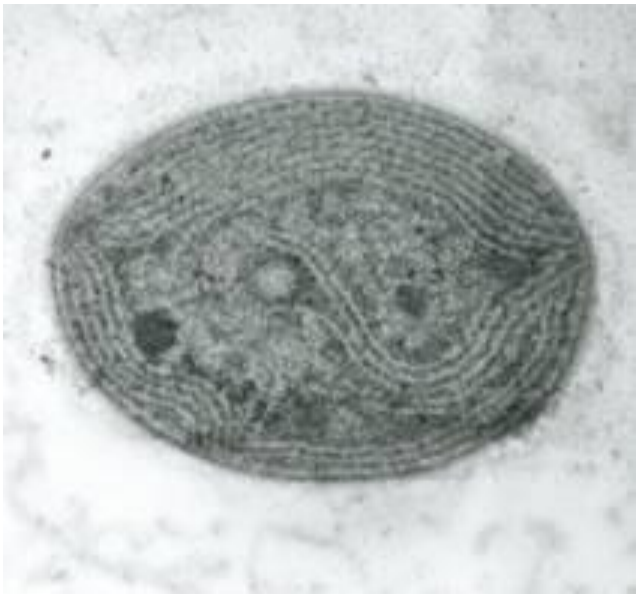


Fig. 1: *Synechocystis* sp. PCC 6803 (Nakamoto, 2014)

Cyanobacteria are gram-negative bacteria. They possess an outer membrane, a cytoplasmic membrane and intracellular membranes, called thylakoid membranes – except Gloeobacteria, which have no intracellular membranes. In cells with thylakoids all chlorophyll can be found these membranes.

Taxonomy of Cyanobacteria

Unicellular as well as multicellular organisms with different shapes, colours and growth modes can be found in the phylum of Cyanobacteria. However the taxonomy of

cyanobacteria is quite a challenge due to the different systems that were used over the time (Oren, 2004).

Nowadays the relation between the different strains of cyanobacteria and their genus is determined by the 16S rRNA sequencing method in combination with stable cytomorphological and ecophysiological markers and ultrastructural and biochemical characteristics (Komarek, 2009).

Currently seven groups of cyanobacteria are defined, that have the following relationship according to Bergey's Manual of Systematic Bacteriology (Boone & Castenholz, 2001):

1. Cyanobacteria without thylakoids
    I. Gloeobacteria, e.g. *Gloeobacter*
2. Cyanobacteria with thylakoids
    A) Cyanobacteria without cell differentiation
        II. Chroococcales, e.g. ***Synechocystis***
        III. Pleurocapsales, e.g. *Xenococcus*, *Dermocarpa*
        IV. Prochlorales, e.g. *Prochlorococcus*
        V. Oscillatoriales, e.g. *Oscillatoria*, *Spirulina*
    B) Cyanobacteria with cell differentiation
        VI. Nostocales, e.g. ***Nostoc***, *Anabaena*
        VII. Stigonematales, e.g. *Fischerella*

The groups I to IV contain unicellular, V to VII multicellular cyanobacteria.

Relevant for this work are the totally sequenced strains:

- *Synechocystis* sp. PCC 6803 (Kaneko *et al*., 1996): one of the best known and most used strains in the field of cyanobacteriology;
- *Nostoc* sp. PCC 7120 (Kaneko *et al*., 2001): a multicellular, nitrogen fixing strain that is capable of differentiating heterocysts;

Photosynthesis and respiration in cyanobacteria

Oxygenic photosynthesis is the process of converting light energy into chemical energy by absorbing CO_2 and light and producing ATP, reduction equivalents and sugars.

$$6\ CO_2 + 6\ H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6\ O_2$$

All cyanobacteria are capable of photosynthesis and respiration. The electron transfer chains for both processes share several components such as the cytochrome *bf* complex, the redox carrier quinone (Q) and cythochrome c_6 (cyt c) (Peschek, 1999). In cells with thylakoids the

respiration occurs in the cytoplasmic membrane (CM) and the intracellular membranes (ICM), whereas the photosynthesis is restricted to the ICMs (Vermaas, 2001).

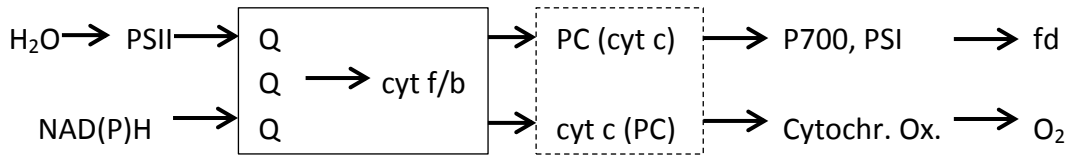


Fig. 2: Shared components of photosynthesis and respiration

Fig. 2 shows the shared components of the electron transfer chain. When enough light is available, the photosynthetic electron transport chain is preferred. At low light or darkness the respiratory rate increases. The redox state of both processes is highly regulated. The photosynthetic electron transport chain in higher plants is essentially identical to the one in cyanobacteria.

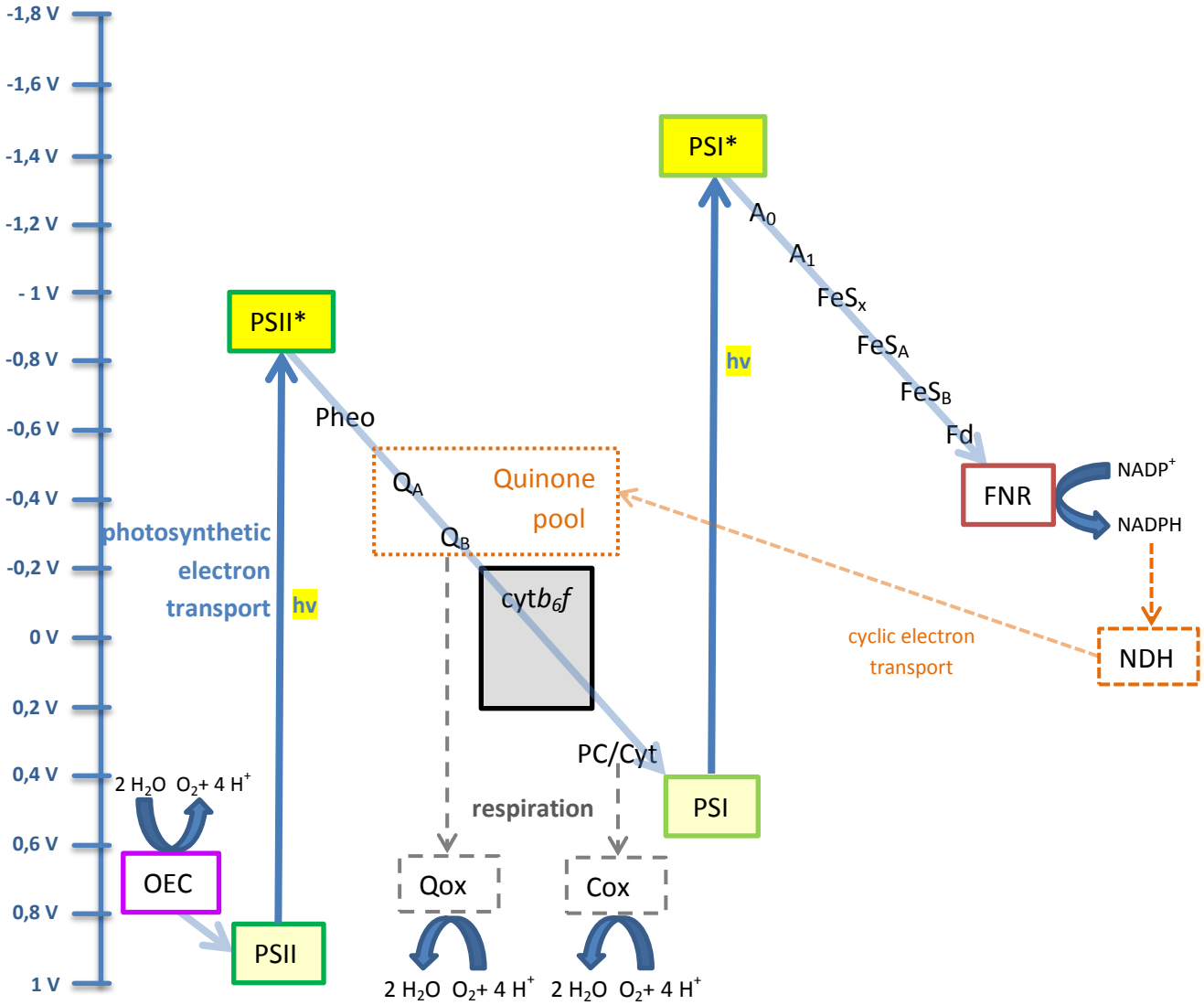


Fig. 3: electron transport scheme of photosynthesis and respiration; OEC = oxygen evolving complex, PS = photosystem, Pheo = pheophytin, Q = plastoquinone, PC = plastocyanine, Cyt c = cytochrome c, A_0 = chlorophyll, A_1 = phyllochinone, FeS = iron cluster, Fd = ferredoxin, FNR = ferredoxin-$NADP^+$-oxidoreductase, NDH = NADPH dehydrogenase, Qox = quinol oxidase, Cox = cytochrome c oxidase

The respiration in cyanobacteria can follow different pathways. Two of them are shown in Fig. 3. Each pathway ends with a respiratory terminal oxidase (RTO). The genomic sequence of different strains revealed their RTOs, as the structure of these enzymes is highly conserved.

Respiratory terminal oxidases

There are three different types of respiratory terminal oxidases in cyanobacteria:

1. Heme copper oxidases
    a. Mitochondrial type cytochrome c oxidases (Cox)
    b. Alternate respiratory terminal oxidases (ARTO)
        i. Type I
        ii. Type II
        iii. Type III
    c. Cytochrome cbb_3 type oxidase
2. Quinol oxidase (Qox)
3. Plastidic terminal oxidase (Ptox)

All known RTOs in cyanobacteria are inhibitable by KCN.

Heme copper oxidases

Mitochondrial type cytochrome c oxidases (Cox)

This mitochondrial type cytochrome c oxidase has been found in nearly every cyanobacterial strain so far and is the main type of oxidase. The genes are organised in the operon c*oxBAC* or *ctaCDE*, encoding the subunits II, I and III. The cytochrome c can be oxidised either by PSI or by the Cox (Schmetterer *et al*., 1994). It was shown that a functional Cox is essential for chemo-heterotrophic growth of *Synechocystis* sp. PCC 6803 (Pils *et al*., 1997), *Anabaena variabilis* ATCC 29413 (Schmetterer *et al*., 2001) and *Nostoc* sp. PCC 7120 (Stebegg *et al*., 2012).

Alternate respiratory terminal oxidases (ARTO)

In *Nostoc* sp. PCC 7120 the genes of the ARTO are organised in a single operon like the Cox-genes and are homologous to the sequence of c*oxBAC*. In *Synechocystis* sp. PCC 6803 the *coxB* gene for the putative subunit II is separated from the *coxAC* genes.

Usually the annotation *cox1BAC* is used for the Cox and *cox2BAC*, *cox3BAC* and so on for ARTOs. Even though the sequence is very similar, it can differ in two motives:

- ARTOs can have a different motive at the Mg^{2+} binding site in subunit I (Schmetterer *et al*., unpublished)
- ARTOs can lack the Cu_A binding site in subunit II, which is highly conserved (Howitt & Vermaas, 1998).

There are three subclasses of ARTOs, depending which motive differs from the Cox sequence:

- Type I: ARTO motive in subunit I and II
- Type II: ARTO motive in subunit I
- Type III: ARTO motive in subunit II

The ARTOs of type I and III are not able to oxidise cytochrome c. *Synechocystis* sp. PCC 6803 has solely an ARTO type I. A *coxBAC*$^-$ mutant of *Synechocystis* sp. 6803 was not capable of energising the cytoplasmic membrane after adding the quinol oxidase inhibitor HQNO (Pils & Schmetterer, 2001). These results indicate that cyt c_6 is no donor to ARTO type I. The ARTOs of type I and III may play a role in creation of a proton gradient as the Mg^{2+} binding site is essential for the transmembrane translocation of H^+ (Stebegg, 2011). Kranzler *et al*., 2014 showed that ARTO type I is involved in the reductive iron uptake in *Synechocystis* sp. PCC 6803. The ARTO motive of subunit II is highly conserved among the ARTOs of type I and III.

The Cu-binding site is necessary for an electron transfer from cytochrome c, therefore only the ARTO Type II may have a Cox activity and should react positively with a heart cytochrome c oxidation assay (Schmetterer *et al*., 1994). The exact function of this motive is not yet clear and no knock out mutant has been generated for this gene so far.

Cytochrome *cbb$_3$* type oxidase

This oxidase is not very widely spread among cyanobacteria. It may however play a crucial role in respiration for strains without a Cox like *Trichodesmium erythraeum* IMS101.

Quinol oxidases (Qox)

This oxidase is encoded by the *cydAB* operon. The quinol oxidase is thought to mediate the oxidation of the quinone pool. In the case of an overreduction reduction equivalents can be removed without involving the cyt*b$_6$f* complex (Berry *et al*., 2002). In a mutant of *Synechocystis* sp. 6803 that lacks both Cox and ARTO the respiration rate of the remaining Qox is similar to the wildtype (Howitt & Vermaas, 1998). This oxidase can specifically be inhibited by the substance HQNO (2-heptyl-4-hydroxyquinoline-N-oxide) (Pils & Schmetterer, 2001).

Plastidic terminal oxidases (Ptox)

Ptox in chloroplasts is a KCN insensitive oxidase and encoded in a single gene. In plants the Ptox directly oxidises plastoquinol and has plays a role in the carotenoid biosynthesis and chlororespiration (Carol & Kuntz, 2001). However, it has been shown that in cyanobacteria the Ptox is sensitive to KCN as well (Mikulic, 2013).

Respiratory terminal oxidases (RTOs) in *Nostoc* sp. PCC 7120

The number and types of RTOs present in cyanobacteria differ even among closely related strains. The genomic sequences revealed five respiratory terminal oxidases for *Nostoc* sp. PCC 7120 and three for *Synechocystis* sp. PCC 6803 (Pils *et al*., 1997).

The RTOs in *Nostoc* sp. PCC 7120 are:

- one mitochondrial type cytochrome c oxidase (Cox)
- two ARTOs (type I and II)
- one *bd*-type quinol oxidase (Qox)
- one plastidic-type alternative oxidase (Ptox).

Growth modes of cyanobacteria

The different modes of growth are determined by three characteristics:

1) Type of energy source: how is ATP generated?
   a) Phototrophy: ATP is produced by using light as energy source
   b) Chemotrophy: ATP is produced by using chemical reactions as energy source

2) Type of electron source: where do the electrons for the reduction equivalents come from?
   a) Lithothrophy: the electrons are derived from inorganic molecules, e.g. H_2O, H_2S
   b) Organotrophy: the electrons are derived from organic molecules

3) Type of carbon source: In which chemical form is the carbon supplied to the cell?
   a) Autotrophy: carbon source is a molecule with only one C-atom, e.g. CO_2, CH_3OH, CH_4
   b) Heterotrophy: carbon source is a molecule with more than one covalently bound C-atom, e.g. sugars, amino acids, fatty acids

Many cyanobacteria are obligate photoautotrophs and depend on sunlight and CO_2, but some strains can take up sugars or other organic molecules and use them for mixotrophic or heterotrophic growth (Smith, 1983). Three growth modes are possible in cyanobacteria: photo-litho-autotrophy, photo-organo-heterotrophy and chemo-organo-heterotrophy.

When a cell grows mixotrophically it uses carbon of the heterotrophic source but still fixes CO_2 which leads to an enhanced growth compared to the photo-litho-autotrophic growth mode. One of these strains is *Synechocystis* sp. PCC 6803. This strain can grow mixotrophically and heterotrophically when glucose is added to the medium (Rippka, 1972), (Anderson & McIntosh, 1991).

The cyanobacterium *Nostoc* sp. PCC 7120 is capable of mixotrophic growth on glucose (Yu *et al*., 2011) and fructose, as well as photo-organo-heterotrophic and chemo-organo-heterotrophic growth on high concentrations of fructose (Stebegg *et al*., 2012).

The glucose permease of *Synechocystis* sp. PCC 6803

The gene *sll0771* or *gtr* encodes the glucose permease, a sugar carrier with 12 transmembrane regions. The main substrate for this carrier is glucose, but due to the structural similarity also fructose is transported into the cell via the glucose permease (Flores & Schmetterer, 1986). Whereas *Synechocystis* sp. PCC 6803 can grow photo-heterotrophically on a medium supplemented with glucose (Rippka, 1972), the strain dies when the medium is supplemented with fructose. This antibiotic effect is related to the interaction of fructose with the glucose permease in 6803: mutants of *Synechocystis* with an interrupted *gtr*-gene cannot grow heterotrophically on glucose anymore but are resistant to fructose (Flores & Schmetterer, 1986), (Zhang *et al*., 1989).

The uptake of glucose influences the carbon metabolism of the cell. The storage of sugar as sucrose and glycogen-like polysaccharides and the catabolism are enhanced (Narainsamy *et al*., 2011). Therefore the intracellular concentration of metabolites of the oxidative pentose phosphate pathway and glycolysis is decreased.

Aims of this thesis

Stebegg *et al*., 2012 found out, that *Nostoc* sp. PCC 7120 can grow heterotrophically on high concentrations of fructose. Heterotrophic growth of this strain is not possible, when the Cox is knocked out. *Nostoc* sp. PCC 7120 has five different RTOs and the question arises if other RTOs may also be essential for heterotrophic growth. Therefore several RTO$^-$ mutants were to be generated.

The heterotrophic growth of *Nostoc* sp. PCC 7120 is dependent on high fructose concentrations. Fructose is a polar substance and therefore is thought to enter the cell via a so far unknown carrier. It has been found that *Synechocystis* sp. PCC 6803 *gtr*$^-$ grows on high fructose concentrations as well, while fructose is toxic to the wildtype (Stebegg & Schmetterer, unpublished). Gene analysis revealed several possible sugar transporters with high sequence similarities present in both strains. *Synechocystis* sp. PCC 6803 is naturally competent and can easily be genetically manipulated. Thus this strain was chosen for the generation of the sugar transporter mutants.

Respiratory mutants

Several vectors and mutants that were used in earlier works are available in this lab:

- 7120 *Cox*$^-$
- 7120 *Qox*$^-$
- 7120 *A1*$^-$ *A2*$^-$ (ARTO type I$^-$, ARTO type II$^-$)
- 7120 *Ptox*$^-$
- Knock out vector for Cox
- Knock out vector for Qox
- Knock out vector for Ptox

ARTO type I is thought to play a role in the reductive iron uptake in *Synechocystis* sp. PCC 6803 (Kranzler *et al*., 2014). The function of ARTO type II has not been investigated so far. A vector for the knock out of the ARTO type II (operon *cox2BAC*) needs to be constructed.

In further works the generated mutants may also be characterised regarding their respiratory behaviour to find out, whether ARTO type II has a Cox activity or not.

The aim is:

- Constructing a knock out vector for ARTO type II
- Generating RTO knock out mutants by conjugation

Sugar transporter mutants

As mentioned before, fructose is toxic for *Synechocystis* sp. PCC 6803 but a *gtr*$^{-}$ knock out mutant is resistant to fructose. This mutant is capable of growing on high concentrations of fructose as is the wildtype of *Nostoc* sp. PCC 7120. This would indicate that there might be a common uptake mechanism in *Nostoc* sp. PCC 7120 and *Synechocystis* sp. PCC 6803, which allows heterotrophic growth in both organisms. The two transmembrane proteins and possible sugar transporters *slr1202* and *sll1374* were chosen to be knocked out.

lacF – slr1202

The gene *slr1202* is coding for a protein that is annotated as permease protein of sugar ABC transporter at Cyanobase (Kazusa Institute, 1996) and contains 6 transmembrane regions. The protein of *slr1202* shares a similarity of 51 % and identity of 30 % with the gene *slr0530* (blasted at Cyanobase). The gene *slr0530* was shown to be part of the glucoglycerol transport system in *Synechocystis* sp. PCC 6803 and therefore plays a role in the adaptation of the cell to osmotic stress (Mikkat & Hagemann, 2000). However, it has been shown that a knock out of the *slr1202* had no effect on the uptake of glucoglycerol. The function of this gene and its gene product stays unclear.

melB – sll1374

The melibiose carrier in *E. coli* is thought to enable cotransport of different α- and β-galactosides with H^+, Li^+ or Na^+ (Tsuchiya & Wilson, 1978). At Cyanobase the gene *sll1374* is annotated as probable sugar transporter with 13 transmembrane regions. The gene is annotated as "*melB*" or melibiose permease due to the sequence similarity to a protein from *E. coli* K12. This protein is a melibiose transporter and a sequence identity of 34 % with those of *Synechocystis* was revealed after the sequence was blasted at Cyanobase.

Not much is known about this gene in *Synechocystis* sp. PCC 6803, except the fact that it is an integral transmembrane protein and located in one of the cyanobacterial membranes (Kwona *et al*., 2010). So far, the gene or its gene product have not been characterised at all regarding its function in the bacterium.

The aim is:

- Constructing knock out vectors for *slr1202* and *sll1374*
- Growing sugar transporter mutants of *Synechocystis* sp. PCC 6803 on high fructose concentrations

Methods and material

Cultivation of Bacteria

Cultivation of Cyanobacteria

For growing cyanobacteria BG11-based media were used according to Rippka et al., 1979. *Nostoc* sp. PCC 7120 is grown on BG11-HPA as solid medium and BG11 as liquid medium. *Synechocystis* sp. PCC 6803 is grown on BG11-TS agar as solid medium and on BG11-TS as liquid medium. The petri dishes are filled with 25 mL BG11 agar + antibiotics. The dishes are incubated in a light incubator for a minimum of two weeks at 32°C and a relative humidity of 80 %. For a liquid culture 50 mL liquid medium plus antibiotics or other additives is incubated in an Erlenmeyer flask in a light chamber. The culture is constantly shaken with 200 rpm at 32°C.

Table 1: BG11 and BG11TS according to Rippka et al., 1979

Name	Concentration in stock sol.	Conc. in medium %	Conc. in medium	
Stock I	40 mg/l $K2HPO4*3\ H2O$	0.1 (v/v)%	0.18 mM	BG11
Stock II	75 mg/l $MgSO4*7\ H2O$ 50 mg/l $CaCl2*2\ H2O$ 47. 1500 mg/l $NaNO3$ 100 mL/L A5-traces	1 (v/v)%	0.3 mM 0.3 mM 17.6 mM	
Stock III	1 mg/l Na2MgEDTA 6 mg/l FeNH4 citrate 6 mg/l citric acid	0,1 (v/v)%	2.8 µM 22.8 µM 31 µM	
Stock IV	20 mg/l $Na2CO3$	0,1 (v/v)%	0.19 mM	
A5-traces	0.23 mg/l MoO_3 2.86 mg/l H_3BO_3 1.81 mg/l $MnCl_2*4\ H_2O$ 0.22 mg/l $ZnSO_4*7\ H_2O$ 0.08 mg/l $CuSO_4*5\ H_2O$ 0.05 mg/l $Co(NO_3)2*6\ H_2O$		1.6 µM 46 µM 9.7 µM 0.77 µM 0.32 µM 0.17 µM	
TES-HCl	1 M (pH 8,0)	1 (v/v)%		TS
Na-thiosulfate		0,3 (w/v)%		

BG11-TS is supplemented with bacto agar in a concentration from 1 to 2 % (w/v) for BG11-TS agar. For BG11-HPA, BG11 is supplemented with highly purified agar (bacto agar washed twice with water, once with ethanol and once with acetone) in a concentration of 1 % (w/v). Heating in a microwave can liquefy both media.

Storage of Cyanobacteria

Cyanobacteria can be stored at room temperature without direct light on agar for months. The petri dish has to be sealed with a parafilm to prevent drying of the solid medium.

For long time storage a 50 mL liquid culture is centrifuged in a 50 mL Falcon tube at 3000 rpm for 5 – 10 minutes (depending on the strain) at room temperature. The supernatant is removed and the pellet is resuspended in 20 mL liquid medium. The solution is centrifuged as described before and the supernatant is removed again. 1 mL of the remaining liquid pellet is transferred into a Nunc tube. 300 µL DMSO are added. The cell suspension is vortexed and shock frozen as quickly as possible in liquid nitrogen. The cryo-culture can be stored at -80°C for decades.

Control for axenic liquid cultures

Axenity is an important issue when cyanobacteria are grown. The selection on certain characteristics is adulterated when the culture is contaminated. Different controls can be run when there is a suspicion of a xenic culture:

- Stereomicroscopic control: The petri dishes are investigated under the stereomicroscope.
- Microscopic control: A drop of a cell culture is put on an object holder and investigated with a light microscope.
- Microbiologic control: 100 µL of the liquid culture is distributed on a petri dish filled with LB agar and then incubated at 32°C. No colonies should grow when the culture is axenic.
- Quick-test for solutions: some µL solution e.g. medium, sugar or antibiotic solution are mixed with 3 mL LB and incubated at least over night at 37°C.

Cultivation of *Escherichia coli*

As solid medium 25 mL LB agar medium and antibiotics in a petri dish are used to grow *E. coli*. The cells are distributed over the agar and the plate is incubated over night at 37°C. For a liquid culture the cells are grown in 3 to 6 mL LB medium with antibiotics if necessary. The

suspension is incubated for 6 to 16 hours (depends on purpose). For larger plasmid preparations the cells are grown in 30 to 50 mL LB.

Table 2: Media for E. coli: LB and LB agar

5 g/L bacto-tryptone	**LB**	**LB agar**: The LB agar can be liquefied by heating it up in a microwave.
10 g/L yeast extract		
10 g/L NaCl		
1 – 1,5 % (w/v) bacto agar		

For preparing *E. coli* for transformation, the cells are grown in SOB. After the transformation the cells are grown in SOC.

Table 1: The media SOB and SOC

SOB	**Chemicals**	**SOC**
20 g/L	tryptone	20 g/L
5 g/L	yeast extract	5 g/L
0,5 g/L	NaCl	0,5 g/L
10 mM	$MgSO_4$ (stock 1M)	10 mM
-	$MgCl_2$ (stock 1M)	10 mM
-	glucose (stock 1M)	20 mM

The solutions of $MgSO_4$ and $MgCl_2$ are autoclaved separately. The glucose solution is filtered through a sterile filter.

Storage of *Escherichia coli*

3 mL liquid culture of *E. coli* is grown in LB over night (12 to 16 hours). 1 mL of the cell suspension is transferred into a Nunc tube and is mixed with 0.5 mL 50 % glycerol. The mixture is vortexed and then shock frozen in liquid nitrogen. It is stored at -80°C.

Used antibiotics

Concentrations used for *E. coli*, see table below; concentrations for cyanobacteria: see 'Results';

Table 2: Used antibiotics for E. coli

Name	Stock solution	Concentration for *E. coli*
Ampicillin	5 mg/mL in water	50 µg/mL
Erythromycin	50 mg/mL in ethanol	6-10 µg/mL
Gentamycin	1 mg/mL in water	30 µg/mL
Kanamycin	5 mg/mL in water	50 µg/mL
Neomycin	5 mg/mL in water	-
Spectinomycin	10 mg/mL in water	-
Streptomycin	5 mg/mL in water	50 µg/mL

Preparation of DNA from bacterial cultures

Preparation of genomic DNA from cyanobacteria

A liquid culture (25-50 mL) is centrifuged in a 50 mL-Greiner tube with 5.500 rpm at 20°C for 5 minutes (filamentous strains e.g. PCC7120) or 10 minutes (unicellular strains e.g. *Synechocystis* sp. PCC 6803). The supernatant is then removed and the pellet vortexed. The pellet is transferred into a 1.5 mL Eppendorf tube and centrifuged with an Eppendorf centrifuge at room temperature for 2 (filamentous strains) or 10 (unicellular strains) minutes at 8,000 to 10,000 rpms. The supernatant is removed and the pellet is resuspended in 1 mL TE by vortexing. The liquid is centrifuged again as described and the pellet is resuspended in 400 µL TE.

150 µL sterilized glass beads, 10 µL 20 % SDS (10 g/50 mL) and 450 µL phenol/chloroform 1:1 are added. The suspension is vortexed for one minute and then put on ice for one minute. This step is repeated 4 times for filamentous strains and 9 times for unicellular strains. The tube is centrifuged at 4°C for at 14,000 rpm for 15 minutes. The supernatant is transferred to a new 1.5 mL Eppendorf tube. Proteins and remnants of the membranes are removed by phenol-extraction (see phenol extraction). Afterwards a sodium acetate-ethanol precipitation is performed (see ethanol precipitation).

For further purification an RNase digest can follow: 10 µL RNase solution (10 mg/mL) is added to the DNA solution and the tube is incubated overnight at 4°C or for one hour at room temperature. Then an ammonium acetate-ethanol precipitation is conducted to remove the nucleotides.

Preparation of genomic DNA from *Synechocystis* sp. PCC 6803 by Xanthogenate method (Tillett & Neilan, 2000)

To improve the DNA extraction efficiency a new method for genomic DNA preparation from *Synechocystis* sp. PCC 6803 was established:

1 mL of a cyanobacterial cell culture is harvested by centrifugation at room temperature for 10 minutes at 14,000 rpm in a 1.5 mL Eppendorf tube. The supernatant is removed and the pellet is resuspended in 50 µL TER solution (10 mM tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0; 100 µg/mL RNase A). 750 µL freshly prepared XS buffer is added.

XS buffer:

- 1% potassium ethyl xanthogenate;
- 100 mM Tris-HCl, pH 7.4;
- 20 mM EDTA, pH 8.0;
- 1 % SDS;
- 800 mM ammonium acetate

The tube is inverted several times and incubated for 60 minutes at 70°C. The tube is vortexed briefly and placed on ice for 30 minutes. The precipitate is removed by centrifugation for 10 minutes at 14,000 rpm. The supernatant is transferred into a new Eppendorf tube and 750 µL isopropanol are added. The solution is incubated at -20 °C for 10 minutes and then centrifuged for 10 minutes with 14,000 rpm at 4°C. The supernatant is completely removed and the tube is washed with 70 % ethanol. The DNA is dried under vacuum (25 mbar) and dissolved in 50 µL TE (10 mM Tris-HCl pH = 8, 1 mM EDTA pH = 8).

Plasmid preparation from *E. coli* according to Holmes and Quigley, 1981

1.5 to 4.5 mL of an overnight liquid culture are centrifuged at room temperature for 1 minute at 14,000 rpm with an Eppendorf centrifuge in a 1.5 mL Eppendorf tube – when more than 1.5 mL are used, the centrifugation step is repeated. The supernatant is removed with a pipettor and 350 µL STET (8 % w/v sucrose, 5 % v/v Triton X-100, 10 mM Tris-HCl pH = 8, 50 mM EDTA pH = 8) are added. The pellet is resuspended carefully with a pipettor and

25 µL lysozyme solution (10 to 20 mg/mL) are added. The tube is inverted several times and incubated for 5 to 10 minutes. The tube is immersed into a boiling water bath for 45 seconds. The proteins are denaturated in this step and the suspension should turn dull white. The tube is put on ice for a minute and then centrifuged at 4°C for 20 minutes with 14,000 rpm. The rubbery pellet is removed with a sterile tooth pick. 350 µL of 2-propanol are added, the tube is inverted several times and cooled at -80°C for 20 minutes or at -20°C for at least one hour or overnight.

The DNA is precipitating. The tube is centrifuged at 4°C for 30 minutes with 14,000 rpm. The supernatant is removed completely with a pipettor and 500 µL 70 % ethanol are added. The tube is vortexed and centrifuged again for 1 minute. The supernatant is removed completely with a pipettor and the DNA pellet is dried at 25 mbar for 10 minutes. Afterwards the pellet is dissolved in 30 to 50 µL TE (depending on the size of the pellet) and stored at 4°C.

The solution can be further purified by performing an ethanol precipitation (see ethanol precipitation), a phenol extraction (see phenol extraction) or by using a DNA purification kit.

DNA purification

Phenol extraction

400 µL extraction liquid is added to the solution and vortexed for 30 to 60 seconds. The tube is centrifuged at 4°C for 3 minutes at 14,000 rpm. The supernatant is transferred to a new Eppendorf tube. The solution is extracted with:

1. phenol
2. phenol/chloroform (1:1)
3. chloroform/isoamylalcohol (24:1)
4. chloroform

Ethanol precipitation

The volume of the solution is determined with a pipettor. The ethanol precipitation can be performed with:

- Sodium acetate: X µL (x = 10 % of the determined volume of the solution) 3 M sodium acetate are added.
- Ammonium acetate: X µL (x = 50 % of the determined volume of the solution) 5M ammonium acetate are added.

Twice the volume (volume of the solution + X) ethanol p. a. is added. The tube is inverted a few times and then frozen at -80°C for 20 minutes or at -20°C for at least one hour or overnight. The tube is centrifuged at 4°C for 30 minutes with 14,000 rpm. The supernatant is totally removed with a pipettor and 500 µL 70 % ethanol are added. The tube is vortexed and centrifuged at 4°C for one minute with 14,000 rpm. The supernatant is removed totally with a pipettor and the remaining pellet is dried for 8 to 12 minutes (depending on the size of the pellet) at 25 mbar. 30 to 50 µL of TE are added to the pellet and the tube is stored at 4°C.

Electrophoresis

Depending on the size of the DNA fragments 0.5 to 1 % (w/v) agarose is boiled in a microwave oven in 1x TAE-buffer until everything is dissolved. A UV-active dye may be added (depends on the staining, see 'staining' below). The liquid is poured into a special apparatus where it cools down and becomes solid. Sample slots are formed by adding a comb to the apparatus while the gel is still hot and liquid. The gel is transferred to an electrophoresis chamber and covered with buffer. The samples are mixed with 6x loading dye (1 µL per 5 µL sample) and are applied to the slots with a pipettor.

A voltage between 3.5 and 5.5 V/cm is applied. The loading dye indicates the distance that has been run. When the distance is long enough (depending on the DNA-fragments of interest) the gel is stained or directly analysed with UV-light in a BioRad Gel Imager if the gel has already been stained.

The gel can be used for quantifying DNA by adding a certain amount of a known standard. The relative intensity is calculated by the Image J software, version 1.46r (Schneider *et al*., 2012). If precise quantification is needed e.g. for blunt end ligation the amount of DNA is determined with a NanoDrop.

Table 3: Solutions for electrophoresis

stock solutions	chemicals	final concentration
50x TAE	40 mM tris-acetate pH = 8, 1 mM EDTA	1x
6x Loading Dye, storage at 4°C	25 mM Tris-HCl pH = 8, 50 % (v/v) glycerine, 0.25 % (w/v) bromphenolblue, 0.25 % (w/v) xylene cyanole;	1x

Staining

The gel is either stained with ethidiumbromide or with SybrGreen.

- Ethidiumbromide: After the gel is finished, it is incubated for 20 – 30 minutes in a solution of 1000:1 water and ethidiumbromide (10 mg/mL) and is then washed with water.
- SybrGreen: 1 µL SybrGreen per 100 mL gel is added to the hot and liquid gel before it cools down and gets solid.

Gelextraction (Promega, 2012)

For extracting a DNA-fragment of interest in a gel the gel is sliced with a scalpel and transferred into a 1.5 mL Eppendorf tube. The slice is weighed and Promegas Wizard® SV Gel and PCR Clean-Up System is used for extraction. The Promega Quick-protocol is used with the following modifications:

- Binding of DNA: 2-3 minutes at room temperature
- Elution: 20-50 µL of water is added, depending on the concentration of the DNA on the gel before; sometimes TE was used for elution (more efficient recovery of DNA); 3 minutes of final centrifugation;

Amplifying DNA with Polymerase Chain Reaction (PCR)

The desired DNA fragment is amplified via PCR. For the test of homozygous knock out mutants the same programs as in cloning applications are used but with only half the volume and 20 cycles. A Rotor-Gene Q Thermocycler from Quiagen was used for performing the PCRs in this work.

Table 4: Used primers

Gene	Primer	Sequence
cox2B	cox2B-6 (fwd)	GTA TGT GGT TAT CCT GTG TT
	cox2B-BamHI 2 (fwd)	GGC GGG ATC CGT ATG TGG TTA TCC TGT GTT
	cox2B-5 (bkwd)	GAC GTT GTA TGC AAT GTC TC
	cox2B-SalI (bkwd)	CGC GGT CGA CGT TGT ATG CAA TGT CTC
	cox2B-BamHI (bkwd)	CAG TGG ATC CGA CGT TGT ATG CAA TGT CTC

gtr	Gtr-5’ (fwd)	GGG CGG ATT AAA ACG ATG
	Gtr-3’ (bkwd)	GTC ACC AAA GCA ATT ATC CCA GC
lacF	lacF-BspEI (fwd)	ACA ATC CGG AGA GTC TAA GGA ACC GTA AAC TAC
	lacF-BstBI (bkwd)	CAG GTT CGA AGT CCG TTT CAT ACA GTG TTG TC
melB	melB-SalI (fwd)	GTC GAC GAT GCC ATC AAT GAC CCG ATC ATT GGC
	melB-EcoRI (bkwd)	AAC TGG AAT TCC TGC CTG GTT ATT GG

Programs:

Table 5: Program for amplification of the gene alr2514/cox2B from genomic DNA (1742 bp/3457 bp incl. SmR)

µL	Substance	Temp. in °C	Time	Cycles
34	H_2O dest.	98	10 min	-
10	Phusion HF buffer 5x	98	1 min	20 – 30x
1	dNTPs	52	1 min	
1	Primer cox2B fwd	72	2 min	
1	Primer cox2B bkwd	72	10 min	-
1	**Genomic DNA**	10	∞ min	-
1,5	3 % DMSO p. a.			
0,5	Phusion HF			
50				

Table 6: Program for amplification of the gene alr2514/cox2B from plasmidic DNA (3457 bp)

µL	Substance	Temp. in °C	Time	Cycles
34	H_2O dest.	98	2 min	-
10	Phusion HF buffer 5x	98	15 sec	20 – 30x
1	dNTPs	58	30 sec	
1	Primer cox2B fwd	72	2 min	
1	Primer cox2B bkwd	72	10 min	-
1	**Plasmid-DNA**	10	∞ min	-
1,5	3 % DMSO p. a.			
0,5	Phusion HF			
50				

Table 7: Programs for amplification of the gene sll0771/gtr (863 bp/2360 bp incl. EmR)

µL	Substance	Temp. in °C	Time	Cycles
19,5	H_2O dest.	94	5 min	-
2,5	Thermopol Buffer 10x	94	30 sec	20 – 30x
0,5	dNTPs	55-48	30 sec	
0,5	Primer Gtr-5’	72	180 sec	
0,5	Primer Gtr-3’	72	7 min	-
1	DNA	10	∞ min	-
O,5	Taq Polymerase			
25				

Table 8: Program for the amplification of the gene slr1202/lacF (1183 bp/2298 bp incl. KmR)

µL	Substance	Temp. in °C	Time	Cycles
40,5	H_2O dest.	94	5 min	-
5	Thermopol Buffer 10x	94	30 sec	20 – 30x
1	dNTPs	60	30 sec	
1	Primer lacF-BspEI (fwd)	72	210 sec	
1	Primer lacF-BstBI (bkwd)	72	7 min	-
1	DNA	10	∞ min	-
0,5	Taq Polymerase			
50				

Table 9: Program for the amplification of the gene sll1374/melB (1396 bp/3166 bp incl. SmR)

µL	Substance	Temp. in °C	Time	Cycles
40,5	H_2O dest.	94	5 min	-
5	Thermopol Buffer 10x	94	30 sec	20 – 30x
1	dNTPs	56	30 sec	
1	Primer melB-SalI	72	210 sec	
1	Primer melB-	72	7 min	-
1	DNA	10	∞ min	-
0,5	Taq Polymerase			
50				

Test for homozygous genes

The test whether a gene in the genome of a cyanobacterium is knocked out homozygously can be performed with PCR. Due to the deletion and insertion of gene sequences, the PCR products from the knocked out genes differ in size compared to the wildtype genes.

After the transfer of DNA (and the purification of xenic organisms) a single cell colony is used to inoculate a liquid culture. After it has reached an optical density of 1 to 3, a new liquid culture is inoculated and the genomic DNA is obtained as described before. A PCR is performed with the particular program and analysed on an agarose gel.

Modifying DNA

Restriction enzyme digests

DNA, such as isolated plasmids, DNA fragments and PCR products, can be cut by restriction enzymes. All enzymes used were from New England Biolabs (NEB). A usual solution for digestion has 15 to 40 µL total volume and contains:

- 10 ng – 1 µg DNA, optimum are a few 100 ng
- Appropriate buffer (NEB-system), 1 µL 10x buffer per 10 µL H_2O
- One or two restriction enzymes, 2 – 5 U per µg DNA
- Sterile water

The concentration of DNA is estimated by gel electrophoresis (*via* comparison with standards) or spectrometric methods, mainly NanoDrop. Incubation time and temperature depend on the enzyme(s) and are in most cases one hour at 37°C in a water bath.

The DNA fragments have to be purified after digestion:

- by gel electrophoresis: one or several fragments should be separated from each other
- by Wizard® SV Gel and PCR Clean-Up System: very small fragments (only a few base pairs long) cannot bind to the column and are removed

Blunting

A blunting reaction can be carried out when an enzymatic reaction produces sticky ends but blunt ends are necessary for further cloning or ligation. The solution for blunting reactions contains:

- a few hundred ng DNA
- 1 µL NEB buffer 2 10x per 10 µL solution
- 1 µL dNTP 10x per 10 µL solution (desoxy-nucleotides)
- 1 u (max. 2 u) per µg DNA polymerase that can be used for removal of 3' overhang and fill-in of 3' recessed (5' overhang)
- Sterile water

The solution with a total volume of 20 µL is incubated at 25°C for 15 minutes. To stop the reaction 100 mM EDTA is added to a final concentration of 10 mM and the solution is heated for 20 minutes at 75°C. The DNA is purified by gel electrophoresis.

Deep Vent DNA Polymerase and DNA polymerase I, Large Klenow Fragment (both from NEB) were tested and a result could be achieved for both. Due to the easier handling the DNA polymerase I, Large Klenow Fragment was preferred in most cases.

Ligation

Purified DNA fragments can be ligated together. The solution with a total volume of 10 µL contains:

- DNA: between 1 and 10 ng/µL in total
- 1 µL per 10 µL 10x T4 ligation buffer (thawed on ice)
- 1 µL T4 DNA ligase (400 units/mL)

The rate of vector to insert should be 1:3 to 1:5. For a sticky end ligation the solution is incubated at room temperature for one hour, for a blunt end ligation two hours or at 16°C over night. After incubation the solution can be purified with gel electrophoresis but in most cases there is not enough DNA available for detection on the gel. Some µL of the solution are directly used for transformation and the rest is frozen and stored at -20°C.

Transferring DNA

Transformation into *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 is naturally competent and is therefore enables transformation without special preparation.

1.5 mL of a liquid culture of PCC 6803 or a derivative of PCC 6803 is transferred into a 1.5 mL Eppendorf tube. The tube is centrifuged at room temperature for 10 minutes with 14.000 rpm. The supernatant is removed with a pipettor and the pellet is resuspended in 1 mL

BG11-TS by vortexing. The optical density of the cell suspension is measured at 730 nm. The suspension is diluted with BG11-TS until an OD of 2.5 is reached.

150 µL of this suspension are transferred to a 15 mL Falcon tube. 0.5 to 2 µL of transformable DNA is added and the tube is centrifuged briefly at 3.000 rpm (the cells should not sediment). The tubes are incubated in a light chamber at 32°C for 12 to 16 hours. Afterwards the suspension is distributed on a BG11-TS agar plate and is incubated at 32°C in the light for 24 to 48 hours. The cells are washed from the petri dish with 1 to 3 mL of BG11-TS. The suspension is transferred into a 1.5 mL Eppendorf tube and centrifuged at room temperature for 10 minutes with 14.000 rpm. The supernatant is removed, the pellet is resuspended by vortexing and the cells are distributed on a selective BG11-TS agar plate (containing e.g. antibiotics). The petri dish is incubated as described in 'Cultivation of cyanobacteria'.

Conjugation of *Nostoc* sp. PCC 7120 (Wolk *et al.*, 1984)

Since *Nostoc* sp. PCC 7120 is not naturally competent and cannot be prepared for transformation, a conjugative gene transfer has to be performed. A sequence is cloned onto a suicide vector not reproducing in *Nostoc* sp. PCC 7120 and containing the *sacB* gene. The cloned gene is manipulated by inserting an antibiotics resistance cassette. The conjugative plasmid RP4 mediates the conjugation into other bacteria. The coconjugative plasmid pRL528 encodes a nickase and is necessary to perform a conjugation as well.

A donor strain of *E. coli* contains RP4 and an acceptor *E. coli* strain contains the suicide vector. The coconjugative plasmid pRL528 has to be present as well either in the donor strain or the acceptor strain (all three plasmids have to be used). RP4 is conjugated into the acceptor so that all three plasmids are present in one strain. This strain is mixed with the cyanobacterial strain and all plasmids are transferred. None of the three plasmids can reproduce in *Nostoc* sp. PCC 7120 and are therefore lost after a short period of time. If the gene has integrated into the genome, the cyanobacterium is resistant to the antibiotic.

Practical part:

Some mL liquid culture of *E. coli* HB101 containing RP4 and the *E. coli* strain with the suicide vector are grown over night in LB + antibiotics each. One of the two strains contains pRL528 as well. The next day 750 µL of the liquid cultures are centrifuged at room temperature for one minute at 10,000 rpm. The supernatant is removed and the pellet is carefully resuspended with a pipettor in 1 mL LB and centrifuged as before. The supernatant is removed, only a few µL around the pellet remain. The pellet is resuspended in the remaining supernatant and the *E. coli* cells with the different vectors are mixed together. The mixture is incubated for 1 to 1.5 hours at room temperature.

1.5 mL liquid culture of *Nostoc* sp. PCC 7120 is centrifuged for 5 minutes at room temperature with 14,000 rpm. The pellet is washed with 1.5 mL BG11. The pellet is resuspended in 50 µL BG11. A 1:10 and a 1:100 dilution with BG11 are prepared. A sterile nitrocellulose filter is placed on a BG11-HPA plate. 20 µL of each suspension are streaked on the filter in lines. 2 µL *E. coli* mixture are pipetted onto each cyanobacteria dilution. The plate is incubated for 2 days in permanent light at 32°C and 80% relative humidity. Then the filter with the bacteria is transferred to a new plate BG11-HPA, which contains appropriate antibiotics. After 2 to 3 weeks the cyanobacteria that have not been conjugated should be dead and colonies of the resistant cyanobacteria should have grown. These colonies are transferred directly onto a new plate with antibiotics. The cyanobacteria are transferred to new plates until they are axenic and then used to inoculate a liquid culture.

Selection for double recombination (Cai & Wolk, 1990)

100 µL of a liquid culture are applied to a solid BG11-medium that contains 5 % sucrose. Due to the *sacB* gene (only vectors containing the *sacB*-gene are used for conjugation) the cyanobacterium that contains the singly recombined vector is sensitive to sucrose or rather to a metabolic product of sucrose. The vector with the *sacB* gene is lost when a double recombination occurs, as shown in Fig. 4. The bacterium loses its sensitivity to sucrose.

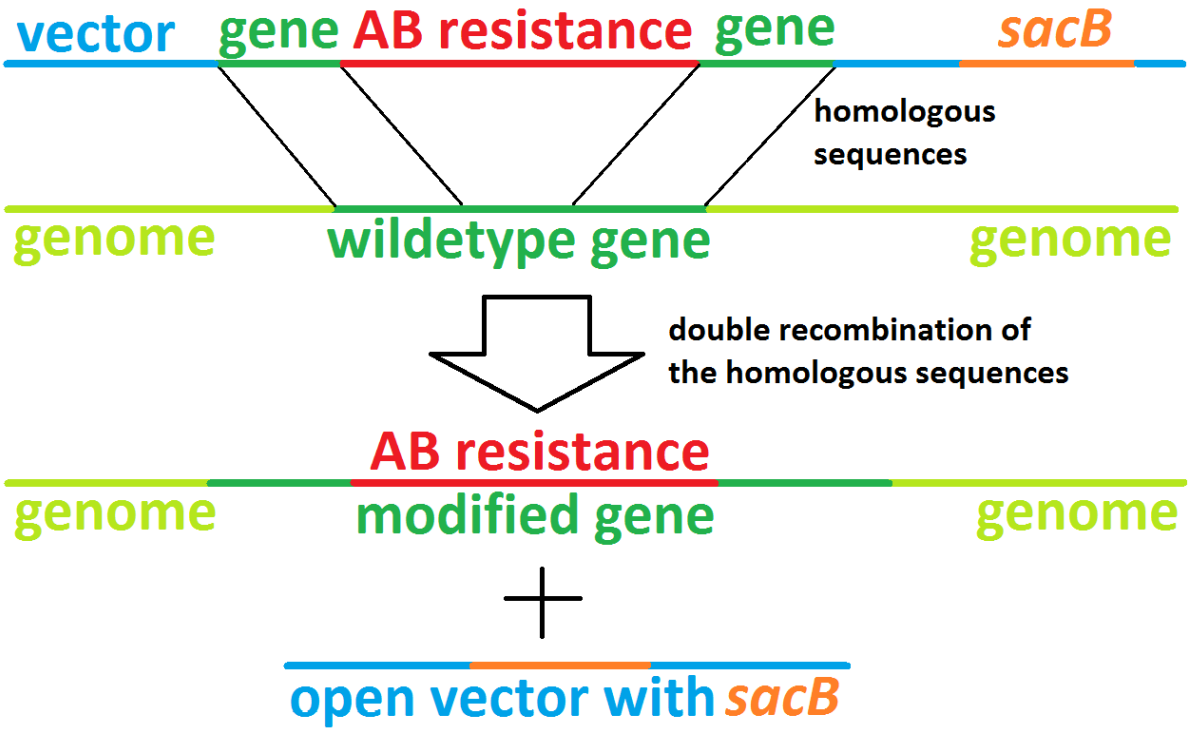


Fig. 4: Double recombination of a suicide vector into the genome

The bacterium stays sensitive to sucrose in case of a single recombination, as can be seen from Fig. 5. The loss of the vector by a second recombination is necessary for the survival of the cell when sucrose is added to the medium.

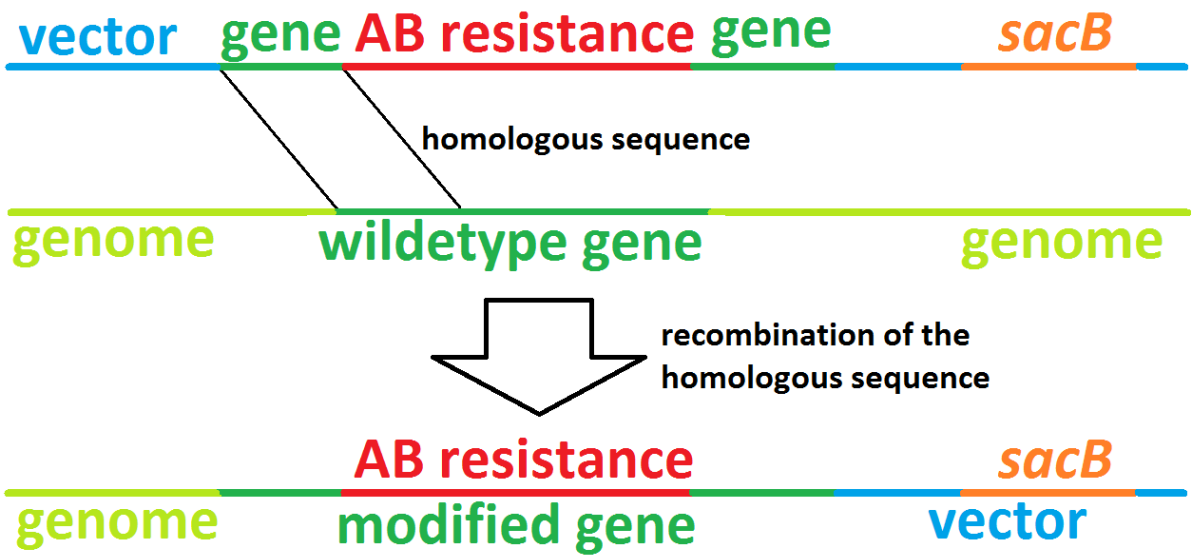


Fig. 5: Single recombination of a suicide vector into the genome

Transformation in *Escherichia coli*

Two different methods where used to transform DNA into *E. coli*:

- Electroporation
- Heat shock

Preparing *Escherichia coli* for transformation

Escherichia coli DH5α, Top10 or HB101 are grown on a petri dish with LB agar. One colony is used to inoculate a 3 mL liquid culture which is then incubated overnight (see 'Cultivation of *Escherichia coli*'). A sterile 1 L Erlenmeyer flask is filled with 200 mL SOB medium. The 3 mL overnight culture is added to the 200 mL SOB medium and the flask is incubated in a shaker at 37°C for one hour at 170 rpm. From now on every 30 minutes the optical density at 550 nm is checked. The intervals may get shorter when the OD_{550} is near the optimum density.

- **Preparing competent cells for Electroporation:**
  When the optimum of OD_{550} = 0.7 to 0.8 is reached the cell suspension is put on ice for 15 minutes. The suspension is transferred to a precooled 250 mL centrifuge tube.

The tube is centrifuged at 4°C for 5 minutes at 5000g. The supernatant is removed and the pellet is resuspended by vortexing. 200 mL of 4°C cold sterile 15 % glycerol (in H_2O) is added. This step is repeated.

The suspension is centrifuged at 4°C for 5 minutes at 5000 g and the supernatant is removed. The pellet is resuspended by vortexing. 60 µL aliquots are either used for immediate transformation or 70 µL aliquots of the suspension are transferred to 1.5 mL Eppendorf tubes and are frozen with liquid nitrogen. They can be stored at -80°C.

- **Preparing competent cells for the heat shock method:**
  When the optimum of OD550 = 0.4 to 0.6 is reached the cell suspension is put on ice for 15 minutes. The suspension is transferred to a precooled 250 mL centrifuge tube.

  The tube is centrifuged at 4°C for 10 minutes at 5000 g. The supernatant is removed and the pellet is resuspended by vortexing. 10 mL of 4°C cold 100 mM $CaCl_2$ is added and the suspension is incubated on ice for one hour. This step is repeated.

  The tube is centrifuged at 4°C for 5 minutes at 5000 g. The supernatant is removed and the pellet is again resuspended by vortexing. 2 mL of 4°C cold 100 mM $CaCl_2$ + 15 % (v/v) glycerol is added. The suspension is incubated for 30 minutes on ice. Eventually 50 µL aliquots are used for transformation immediately or 100 µL aliquots of the suspension are transferred to 1.5 mL Eppendorf tubes and are frozen with liquid nitrogen. They can be stored at -80°C.

Electroporation

A 70 µL aliquot of electro competent cells is thawed on ice for 5 minutes or freshly prepared electro competent cells are put on ice. 1 to 2 µL of a solution containing the DNA to be transformed is added. 60 µL of the suspension are transferred into a precooled (-20°C) electroporation cuvette. The cuvette is put into a cuvette holder which is also precooled at -20°C. The cuvette holder is placed into the electroporator. The electroporation is performed with the following parameters:

- 2,5 kV
- 200 Ω
- 25 µF

The time constant should be between 4.6 and 8 ms. As soon as possible after the shock 1 mL SOC medium is added to the cells. The mixture is transferred to a sterile growth tube and incubated in a shaker at 37°C for one hour at 175 rpm.

The suspension is transferred to a 1.5 mL Eppendorf tube and centrifuged at room temperature for one minute at 10.000 rpm. The supernatant is removed and the cells are

resuspended by vortexing. The cells are distributed on a petri dish filled with 25 mL selective LB agar (containing appropriate antibiotics). The dish is incubated at 37°C over night. The colonies can be checked by plasmid preparation.

Heat shock

Either 100 µL aliquots of chemo competent cells are thawed on ice for 5 minutes or freshly prepared chemo competent cells are put on ice. When the suspension has thawed, 1 to 3 µL solution containing the DNA to be transformed are added. The suspension is incubated on ice for 30 minutes. The cells are heat shocked by applying them to a 42°C warm water bath for 40 seconds. The cells are again incubated on ice for 5 minutes. Then 1 mL SOC medium is added. The suspension is transferred to a growth tube and is incubated in a shaker at 37°C for one hour at 175 rpm.

Afterwards the suspension is transferred to a 1.5 mL Eppendorf tube and centrifuged at room temperature for one minute at 10,000 rpm. The supernatant is removed and the cells are resuspended by vortexing. The cells are distributed on a petri dish filled with 25 mL selective LB agar (containing appropriate antibiotics). The dish is incubated at 37°C overnight. The colonies can be checked by plasmid preparation.

Blue white screening (Ullmann & Perrin, 1970)

When a PCR-amplified DNA is first cloned into a vector there is no possibility of selecting for vectors with integrated insert. Therefore a blue white screening (BWS) can be performed. The used strain of *E. coli* and the vector have to be capable for the BWS.

- genotype bacterium: lacZΔM15 (modification on the F'-plasmid)
- genotype vector: lacZ

The modified bacterium lacks the α-complement of the β-galactosidase. The rest of the β-galactosidase is encoded on the F'-plasmid and the α-complement on the cloning vector. When the vector is transformed into the bacterium, a functional β-galactosidase can be expressed by the bacterium. The enzyme can use the dye X-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galacto-pyranoside) as substrate and therefore the colonies that can express an intact β-galactosidase turn blue. The empty cells, without a functional β-galactosidase, would turn white but due to the added antibiotics they cannot grow.

In cloning vectors that are capable of BWS, the multiple cloning site is located in the lacZ-gene. When a DNA fragment is cloned into the MCS the lacZ-gene is interrupted. After transformation the colonies that carry the interrupted vector turn white because the bacteria cannot express a functional β-galactosidase. That means the blue colonies carry the empty vectors and the white colonies should carry vectors with inserts.

To use this system the lacZ-gene has to be expressed. Therefore the vector carries the lacZ-promoter which normally is inactive because a lac-repressor (encoded on the F'-plasmid) binds to it. When galactose is added the repressor leaves the promoter. But the activation by galactose would only last a short time. Therefore another galactose derivative that prolongs the activation is used: isopropyl-beta-thiogalactopyranoside (IPTG).

The LB agar plates are prepared with appropriate antibiotics. When they are solid 100 µL of a freshly mixed solution are distributed on each plate:

- 4 µL IPTG (stock: 2g IPTG dissolved 10 mL H_2O, filtered through a sterile filter, aliquoted, frozen and stored at -20°C)
- 40 µL X-Gal solution (stock: 20 mg X-Gal dissolved in 1 mL N,N-Dimethylformamid)
- 56 µL H_2O

The plates are incubated open for 20 minutes to allow the DMF, which is toxic for the bacteria, to evaporate. The cells from the transformation are distributed on the plates and incubated over night at 37°C. The contrast between blue and white colonies can be improved when the plates are incubated at 4°C for 20 to 30 minutes. Some of the white colonies are used to inoculate 3 mL LB each. The cultures are incubated over night at 37°C with permanent shaking at 200 rpm. The cultures can be used to perform plasmid DNA extraction and analyse the insert of the vector.

Used plasmids and strains

Used plasmids from the culture collection of the laboratory

- pUC19: cloning vector containing an Amp^R cassette (Yanisch-Perron et al, 1985)
- pRL463 (donor for Sm^R); (Elhai & Wolk, 1988)
- pRL446 (donor for Km^R); (Elhai & Wolk, 1988)
- pRL278: cloning vector containing *sacB*-gene; (Black *et al*. 1993)
- RP4 (for conjugation); (Pansegrau, 1988)
- pRL528 (for conjugation); (Elhai & Wolk, 1988)
- pALUV 12: pRL25V + *gtr*::Em^R (Ludwig, 2000)
- pRStUV1: pRL278 + 7120 *ptox* right and left flanking sequence, Gm^R (of pBBR1MCS-5) inserted into the gene at XhoI site, cassette parallel to gene; (Stebegg, 2011)
- pRStUV2: pRL278 + 7120 *ptox* right and left flanking sequence, Gm^R (of pBBR1MCS-5) inserted into the gene at XhoI site, cassette antiparallel to gene; (Stebegg, 2011)
- pRStUV3: pRL278 + 7120 *cox1*-gene, Km^R from pRL446 inserted into the gene; (Stebegg, 2011)

Newly constructed plasmids used in this work:

- pEHUV2: pUC19 + 6803 *lacF* inserted between the BspEI and BstBI sites, Km^R from pRL446 inserted into the gene at the BclI site;
- pFDUV2: pUC19 + 6803 *melB* inserted between the sites of BamHI and EcoRI, Sm^R from pRL463 inserted into the gene at HindIII site

Strains used:

E. coli: HB101, DH5α, TOP10

Cyanobacteria:

Table 10: Used strains of Cyanobacteria

Strain	Genotype
6803	wildtype
6803 GS1 (*gtr*$^-$)	*gtr*::Km^R
7120	wildtype
7120 *cox*$^-$	*cox1BAC*::Nm^R
7120 *A1*$^-$ *A2*$^-$	ARTO type I::Nm^R, ARTO2 type II::Sm^R
7120 *qox*$^-$	*qox*::Sm^R

Newly constructed strains used:

Table 11: Newly constructed cyanobacterial strains

Strain	Genotype
6803 *gtr*$^-$	*gtr*::Em^R
6803 *lacF*$^-$	*lacF*::Km^R
6803 *GF*$^-$ (*lacF*$^-$/*gtr*$^-$)	*lacF*::Km^R, *gtr*::Em^R
6803 *melB*$^-$	*melB*::Sm^R
6803 *GB*$^-$ (*melB*$^-$/*gtr*$^-$)	*melB*::Sm^R, *gtr*::Km^R

6803 *BF*$^-$ (*melB*$^-$/*lacF*$^-$)	*melB*::Sm^R, *lacF*::Km^R
6803 *3f* (*melB*$^-$/*lacF*$^-$/*gtr*$^-$)	*melB*::Sm^R, *lacF*::Km^R, *gtr*::Em^R

Spectrometry

Measurements of the optical density

The density of a cyanobacterial suspension can be determined by spectrophotometry at 730 nm (OD_{730}). A suspension of *E. coli* is measured at 550 nm (OD_{550}). The measurements were done with a double beam photometer: one cuvette with medium and one cuvette with suspension are put into the photometer. The light extinction of the sample is determined in comparison to the reference (BG11 or BG11-TS for cyanobacteria, LB for E. coli).

For the measurement the culture is diluted in a plastic semi-micro cuvette 1:1 with medium. When the value of the signal is above 1, the sample is diluted further until a value beneath 1 is reached.

Measurements of the chlorophyll content

The content of chlorophyll is sometimes additionally determined after the measurement of the OD_{730}. For this the suspension of the cuvette is transferred into a 1.5 mL Eppendorf tube and centrifuged at room temperature with 14,000 rpm for 10 minutes. The supernatant is removed and the pellet is resuspended by vortexing. 1 mL methanol is added and the suspension is thoroughly vortexed for some minutes. The suspension is centrifuged at room temperature for 2 minutes at 14,000 rpm. The pellet should be blue now. 100 µL of the extract are transferred to a glass cuvette and mixed with 2 mL methanol. The extinction E at 665 nm is determined with methanol as reference. The content can be calculated using the Beer-Lambert law:

$$E = \varepsilon * c * d$$

$$c = E/(\varepsilon * d)$$

ε = 74.5 L/(g*cm);

d = 1 cm;

c = concentration in g/L;

Growth measurements of *Synechocystis* sp. PCC 6803

Photo-autotrophic growth

A 100 mL flask is filled with 50 mL BG11-TS + appropriate amounts of antibiotics if needed. The OD_{730} is 0.1 or 1. The liquid culture is incubated on a shaker in permanent light at 30°C and an air humidity of 60 %. The optical density is measured every 3 to 4 days.

Mixotrophic growth

10 – 200 mM fructose is added to 50 mL BG11-TS + appropriate antibiotics. The OD_{730} is 0.1 or 1 and the culture is incubated as described in 'photo-autotrophic growth'.

Photo-heterotrophic growth

The substance DCMU (3-(3,4-Dichlorophenyl)-1,1-dimethylurea) inhibits PSII and thus oxygenic photosynthesis. The photo-heterotrophic growth is performed like the photo-autotrophic or mixotrophic growth but additionally 10 µM DCMU are added.

Viable counts

A culture is washed and diluted to an OD_{730} of 0.1. A 1:100 suspension is prepared and 50 µL are distributed over a BG11-TS-Agar plate. The plate is incubated for two to three weeks in a light incubator at 30°C and 80 % humidity. After incubation the colonies are counted.

Sugar uptake measurements in *Synechocystis* sp. PCC 6803

A liquid culture with an OD_{730} from 1 to 5 is transferred to a 50 mL Falcon tube. The suspension is centrifuged at room temperature for 10 minutes at 5.500 rpm. The supernatant is removed and the cells are resuspended in BG11-TS. The desired amount of fructose (0-200mM) is added and the suspension is diluted to an OD_{730} of 3. 10 mL are transferred into a thermostated cell chamber with a magnetic stirrer and a light source. 1 µL of radioactive $U^{14}C$ labelled fructose is added and the uptake begins.

Every hour one 1 mL aliquot of the suspension is transferred onto a Millipore nitrocellulose filter HA with a pore size of 0.22 µm, which is connected to a vacuum pump. The medium is

sucked away and the cells are washed with 5 to 10 mL BG11-TS. The filter is then transferred to a scintillation tube and 10 mL scintillation cocktail are added.

Scintillation cocktail according to Bray, 1960:

- 60g naphthalene
- 4g PPO (2,5-diphenyl-1,3,4- oxadiazole)
- dissolved in 1 l dioxane.

After finishing the measurements the 100 % value is prepared by pipetting 0.5 mL of the remaining suspension of the chamber into a scintillation tube and adding 9.5 mL scintillation cocktail. The same is done with 1 mL of the original suspension and for the blank.

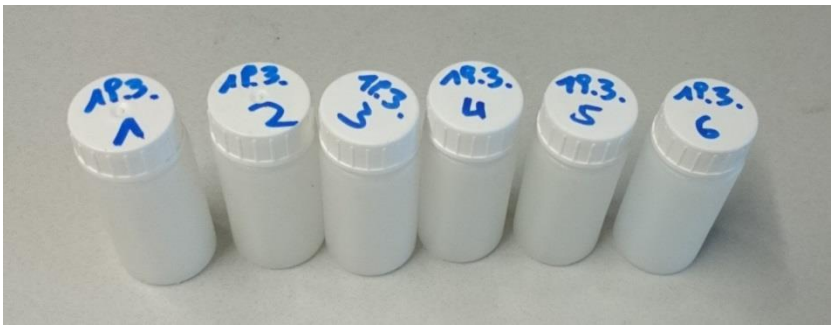


Fig. 6: Tubes used for the radioactivity assay

Results

Part 1: Construction of a knock out vector for ARTO type II and generation of RTO mutants in *Nostoc* sp. PCC 7120

Overview

The gene *alr2514* or *cox2B*, which encodes the subunit II of the ARTO type II in *Nostoc* sp. PCC 7120, was chosen for the construction of the knock out vector. The following steps were made:

- finding a PCR program for *cox2B*
- cloning the gene onto a vector = pEFUV1
- modifying the gene by deleting a sequence and interrupting it with a Sm^R cassette
- cloning *sacB* onto the vector

Furthermore different RTO mutants were generated by conjugation using knock out vectors from earlier works.

A PCR program for *cox2B* of *Nostoc* sp. PCC 7120

The PCR program for the gene *alr2514*/*cox2B* from genomic DNA of *Nostoc* sp. PCC 7120 required special conditions:

- Different polymerases were tested (Pfu Polymerase of Invitrogen, Taq Polymerase of NEB) but only the Phusion® High-Fidelity DNA Polymerase from NEB led to a reproducible PCR product when 3 % DMSO were added to the PCR mix.
- Additives other than 3 % DMSO like increased concentration of $MgCl_2$, DMF or 6 % DMSO did not enhance the yield of the PCR product, as can be seen in Fig.7.
- The first denaturation time had to be increased to 10 minutes otherwise the amount of PCR product decreased.

Fig. 7 shows the agarose gel of PCRs of genomic DNA under different conditions. Sample F with 3 % DMSO showed the highest yield of the desired PCR product. The PCR product of *cox2B* is 1742 base pairs long, for the program and the primers see 'material and methods, Amplifying DNA with Polymerase Chain Reaction (PCR)'.

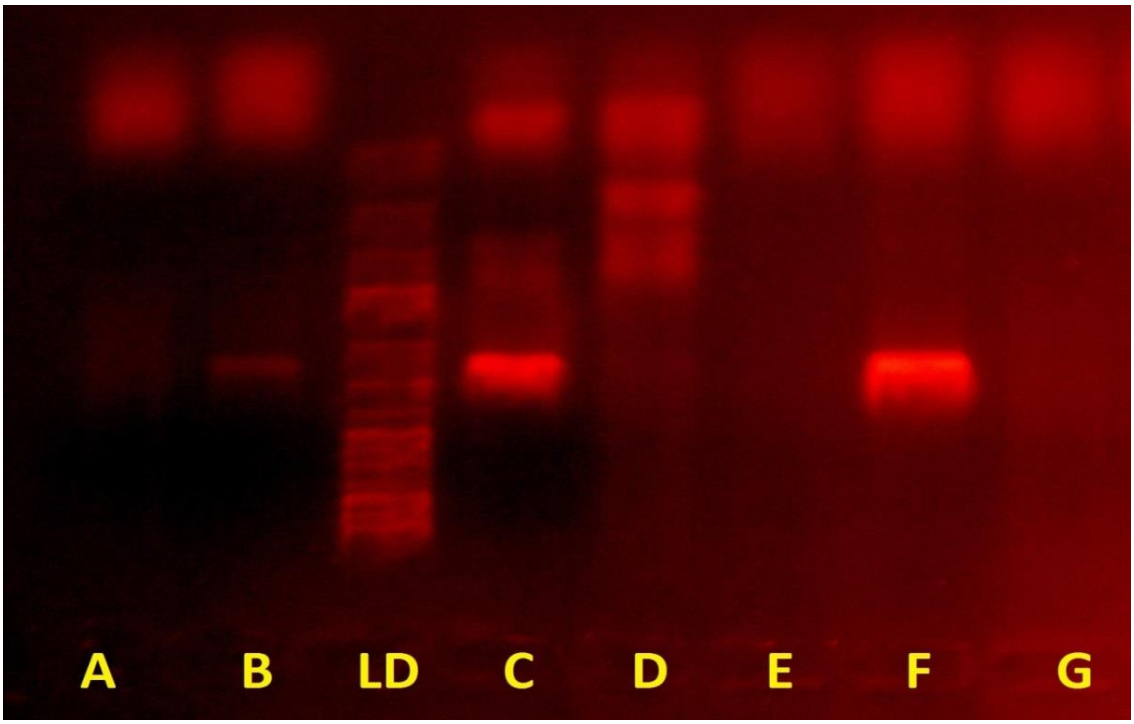


Fig. 7: Agarose gel with PCR products of *cox2B*; A = without additives and 0.5 µL primer, B = without additives with 1 µL Primer, LD = GeneRuler 1 kb ladder ready to use mix, C = 2 M $MgCl_2$, D = 2.5 M $MgCl_2$, E = 0.8 M DMF, F = 3 % DMSO, G = 6 % DMSO

Construction of pEFUV1: cloning *alr2014* into pUC19

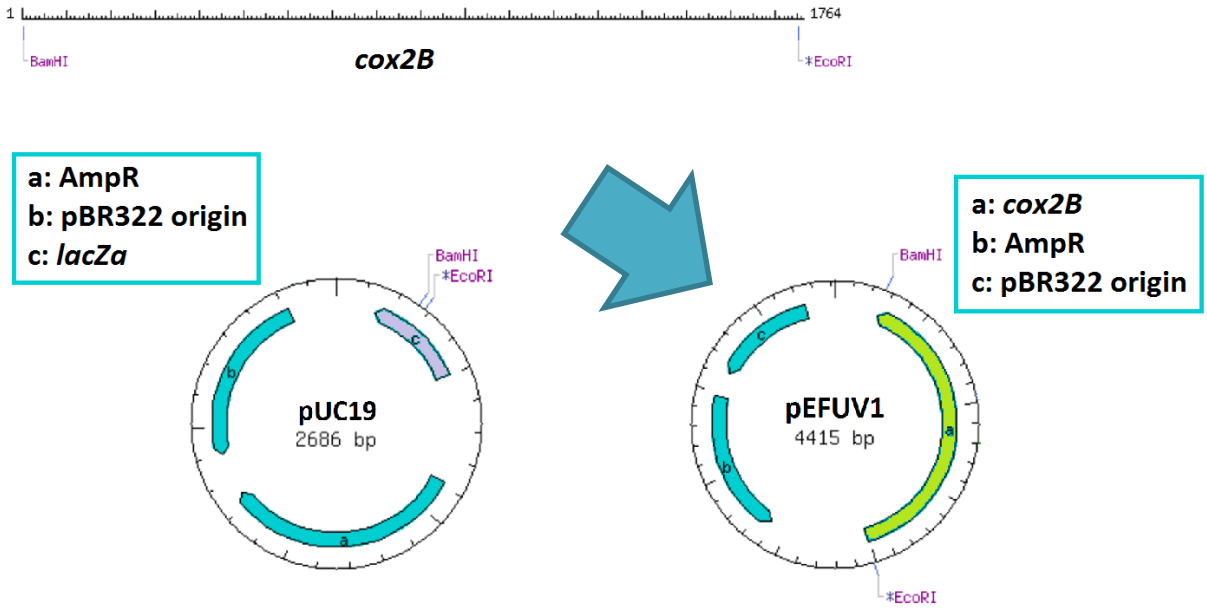


Fig. 8: Construction of pEFUV1

The vector pUC19 was isolated and double digested with BamHI and SalI. The completion of the digest was controlled with ScaI on an agarose gel. A PCR product was obtained, program

as described in ‘material and methods’, with Primers *cox2B*-BamHI and *cox2B*-SalI. The fragments were ligated over night at 16°C as described in ‘material and methods’.

The ligated DNA was transformed via heat shock in *E. coli* DH5α. The bacteria were selected with 50 µg/mL ampicillin and a blue-white-screening was performed, see Fig. 9. The strain TOP10 contains a genomic streptomycin resistance. However a Sm^R cassette was to be inserted into the cloning plasmid and therefore the strain DH5α without genomic antibiotic resistance was chosen for the transformation steps in *E. coli*.

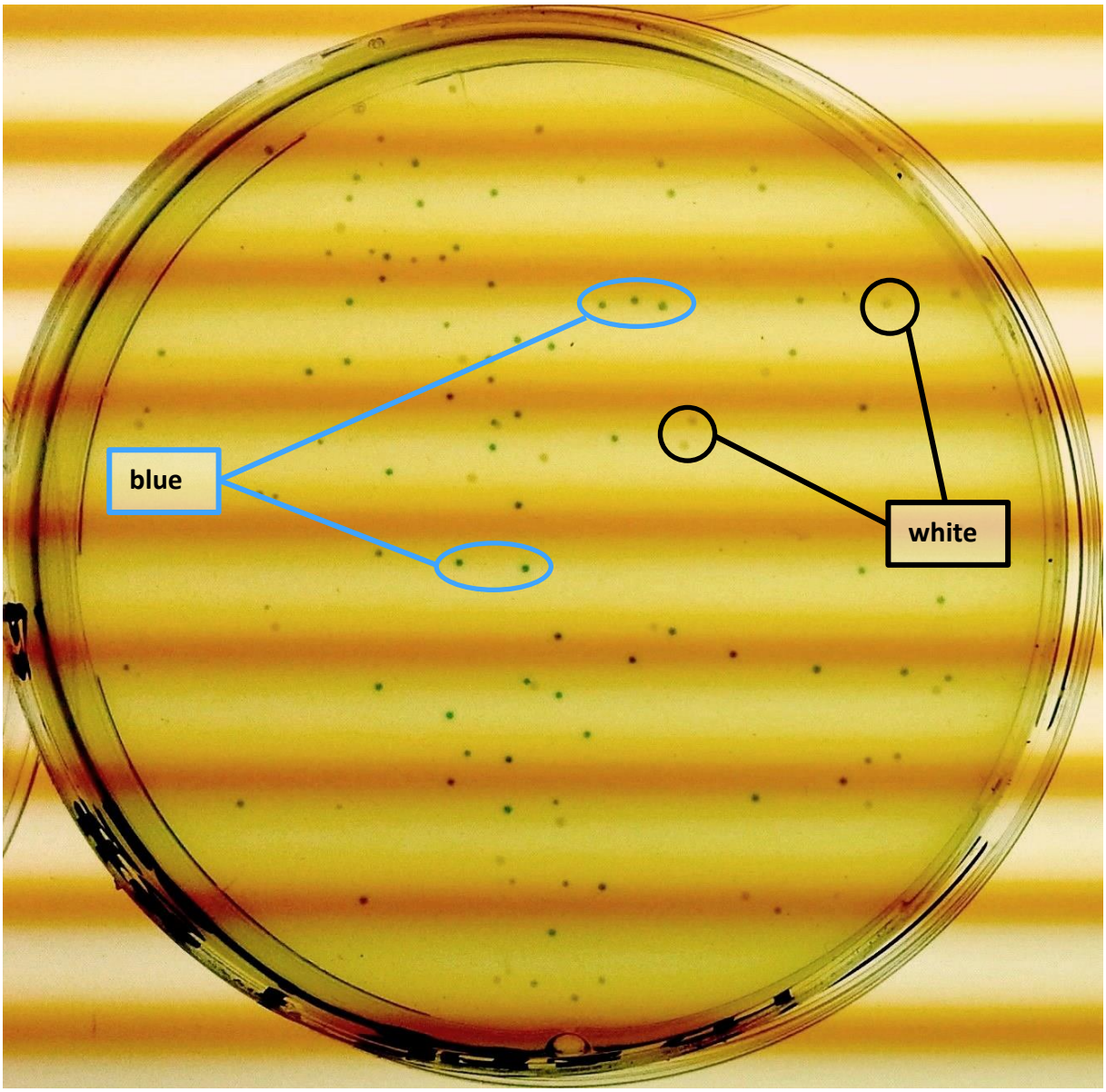


Fig. 9: Blue-white-screening on an LB agar plate

A colony PCR of 18 positive (white) colonies was performed, see Fig 10. The PCR products of a positive colony PCR are 1742 base pairs (bp) long (marked in yellow in Fig. 10) and are compared to a wildtype control (PCR product of genomic wildtype DNA). For a colony PCR the PCR mixture is directly inoculated with a colony moments before the reaction is initiated. 12 of 18 samples each representing one white colony (samples number 1, 2, 3, 4, 7, 8, 9, 10,

11, 15, 17 and 18 in Fig. 10) showed the same size of the PCR fragment as the wildtype control, as can be seen in Fig. 10. Liquid cultures and plasmid preparation were carried out for the 12 positive colonies.

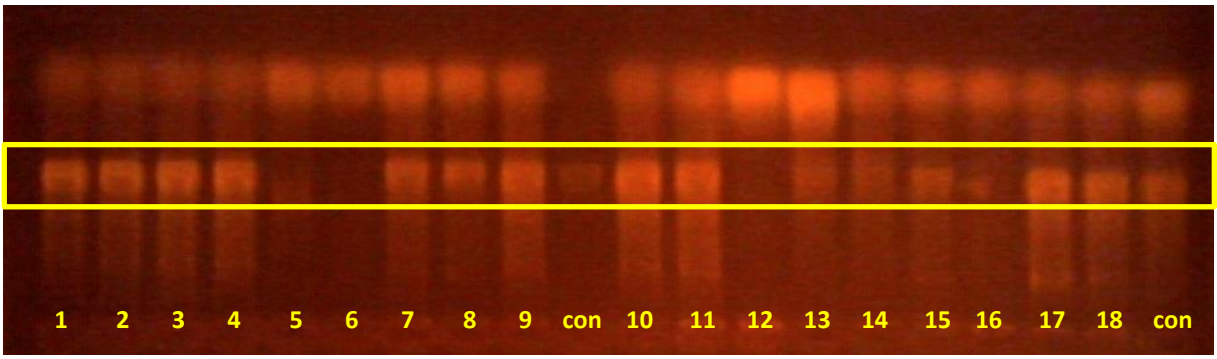


Fig. 10: colony PCR products of 18 positive (white) colonies obtained from the plate with the blue-white-screening assay; 1 = colony named 1, 2 = colony named 2, and so on; con: control = PCR product from genomic DNA

The obtained plasmids were digested with different restriction enzymes each:

Table 14: Fragment pattern for restriction digests of pEFUV1

Enzymes	**Fragment size of pEFUV1 in base pairs**
ScaI	4415
ScaI/EcoNI	1942 2457 16 (not visible on an standard agarose gel)
BamHI/EcoRI	1750 2665

8 of the 12 tested samples showed the expected fragment pattern of pEFUV1. The construction of pEFUV1 was successful. The plasmid in colony number 8 (Fig. 10) was defined as pEFUV1 and the strain shock frozen and stored at -80°C.

Construction of pEFUV2: interrupting *alr2014* on pUC19 with an antibiotic resistance cassette

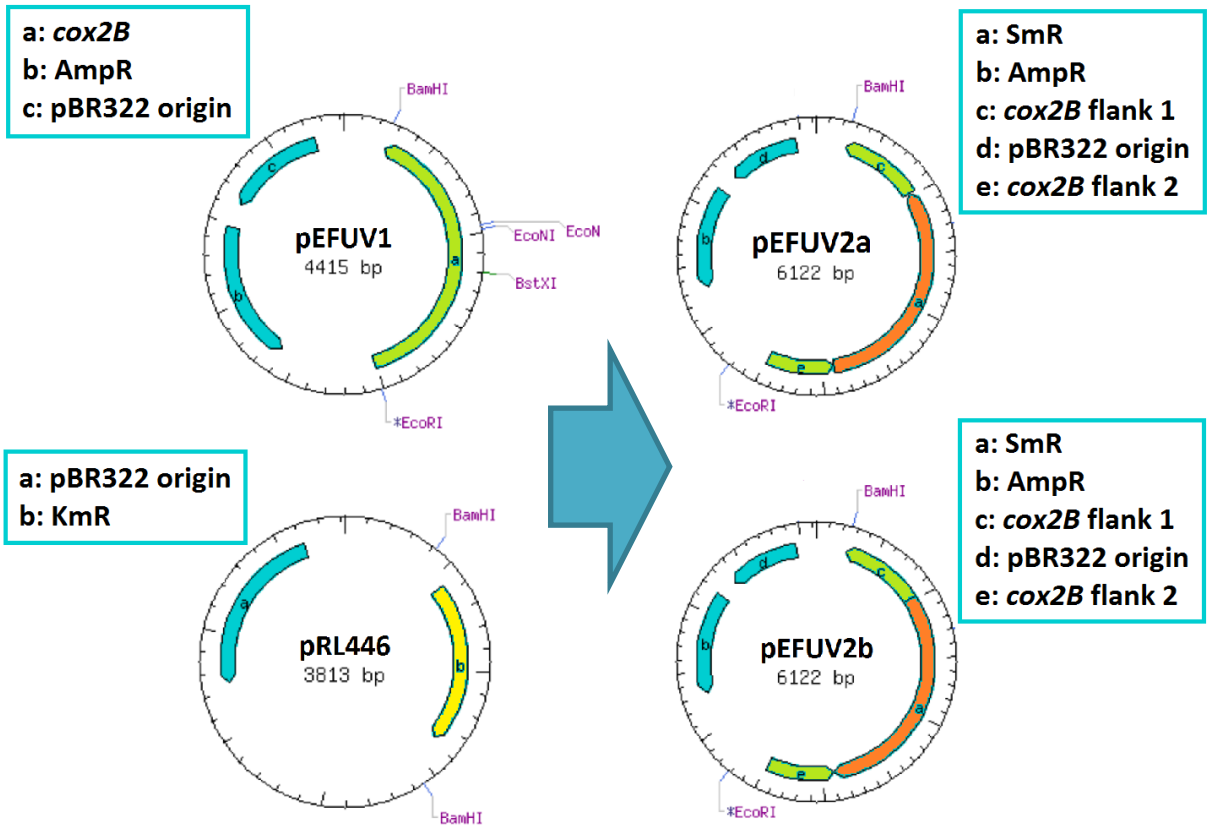


Fig. 11: Construction of pEFUV2a and b

The plasmid pRL463 was isolated from a liquid culture of *E. coli* DH5α (containing the plasmid). The plasmid carries a 2 kbp long streptomycin/spectinomycin resistance cassette which was cut out with DraI, producing blunt ends. The Sm^R-fragment was purified with an agarose gel.

pEFUV1 was isolated and cut with EcoNI and BstXI to remove 235 base pairs in the middle of the *cox2B* fragment. The sticky ends were converted to blunt ends by incubation with a DNA Polymerase I, Large Klenow Fragment from NEB, see 'material and methods, Blunting'. The 4150 bp long EcoNI-BstXI-fragment of pEFUV1 was purified by an agarose gel.

The pEFUV1-fragment and the Sm^R-fragment were ligated over night at 16°C. The mix was transformed by heat shock into *E. coli* DH5α cells and selected on plates with 50 µg/mL ampicillin and 50 µg/mL streptomycin. 13 colonies were obtained the next day and used to inoculate each 3 mL LB medium. Plasmid isolations from these liquid cultures were performed. 8 digests with different enzymes were performed, as can be seen in table 15.

Table 15: Fragment pattern of pEFUV2a and b after digestion with different restriction enzymes

Enzymes	Fragment size of pEFUV1 in base pairs	
	pEFUV2a	pEFUV2b
SalI	6122	6122
BamHI/EcoRI	3457 2665	3457 2665
BamHI/BstXI	6122	6122
XmnI	3246 2876	4218 1904
PvuI	3538 1688 896	3094 2132 896
BglI	2432 1466 1118 1106	2592 1306 1118 1106
AccI	6122	6122
BamHI/EcoRV	5570 552	5570 552

Two samples showed the expected patterns of pEFUV2a and pEFUV2b (agarose gel pictures not shown), which differ in the direction of the inserted Sm^R cassette, as can be seen in Fig. 11. The samples were shock frozen and stored at -80°C.

Construction of pEFUV3: cloning *sacB* onto pEFUV2

For control whether the double recombination of the gene from the vector into the genomic wildtype gene was successful, the *sacB*-gene is a useful tool. Carrying this gene on the vector or the genome makes the bacterium sensitive to sucrose. If the vector acts as suicide vector (as expected) and recombines, the *sacB*-gene is lost and the bacterium is no longer sensitive to sucrose. pRL278 cannot replicate in *Nostoc* sp. PCC 7120, so the only way for the mutation to manifest is to recombine into the genome.

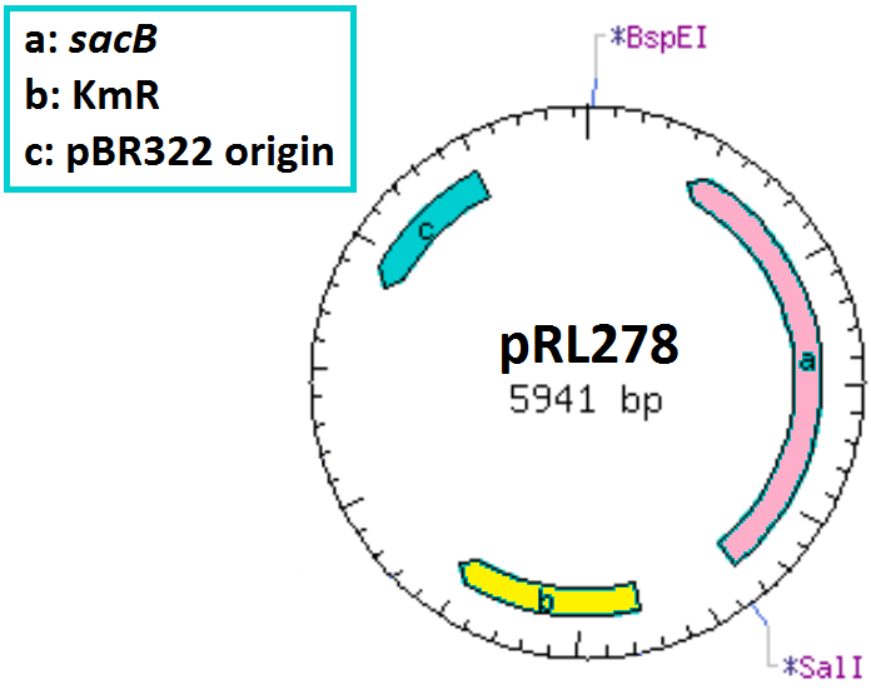


Fig. 12: The vector pRL278

The *sacB* gene of pRL278 was cut out with BspEI and SalI, as can be seen in Fig. 12. First the digest with SalI was performed. The mixture was purified by the Wizard® SV Gel and PCR Clean-Up System and then digested with BspEI. pEFUV2a and b were each digested with PciI and SalI.

The 2345 bp long *sacB* fragment and the 5745 bp long pEFUV2 fragment were purified by an agarose gel, which again revealed a low efficiency digest of pRL278 with SalI. The bands were extracted and ligated together. The subsequent transformation by heat shock led to many colonies. The colonies were used to each inoculate 3 mL LB medium. Plasmid isolations and digests with restriction enzymes were performed and showed that all tested colonies carried the expected plasmid.

To test the functionality of the *sacB* gene some of the colonies were transferred to an LB agar plate containing 50 µg/mL streptomycin and 15 mM sucrose (stock 1.5 M). The plates were incubated over night at 37°C.

All tested colonies could grow, that means the *sacB* gene was not functional in the tested colonies. Spontaneous mutations in the *sacB* gene occur rather frequently and no further attempts were made to obtain pEFUV3.

Conjugation of *Nostoc* sp. PCC 7210 RTO mutants with pRStUV1, 2 and 3

The following strains and plasmids were used to generate the mutants:

Table 16: Strains and plasmids for conjugation

Strain	Plasmid	Expected genotype
7120 *Cox*$^-$	pRStUV1 pRStUV2	7120 *cox*$^-$ *Ptox*$^-$
7120 *Qox*$^-$	pRStUV3	7120 *cyd*$^-$ *Cox*$^-$
7120 *A1*$^-$ *A2*$^-$	pRStUV1 pRStUV2	7120 *Cox23 Ptox*$^-$

The following conjugations were performed as described in 'material and methods, Conjugation':

Table 17: Donors and acceptors for conjugation

***E. coli* strain 1 (donor)**	***E. coli* strain 2 (acceptor)**	**Cyanobacterial strains**
HB101 (RP4)	HB101 (pRStUV1 + pRL528)	7120 *Cox*$^-$, 7120 *A1*$^-$ *A2*$^-$
HB101 (RP4)	HB101 (pRStUV2 + pRL528)	7120 *Cox*$^-$, 7120 *A1*$^-$ *A2*$^-$
HB101 (RP4 + pRL528)	HB101 (pRStUV3)	7120 *Qox*$^-$

The selective plates were incubated for 3 weeks. Especially the cultures of 7120 *A1*$^-$ *A2*$^-$ had to be shadowed due to their increased sensitivity to light. The colonies were streaked onto new plates and incubated again under previous conditions for 2 to 3 weeks. This process was repeated until the culture was free from contaminations and was done 3 times in total and still contained *E. coli* at the end of the thesis.

The cultures of 7120 *A1*$^-$ *A2*$^-$ were difficult to grow on plates and did so only under very mild conditions (lowered concentration of antibiotics and not under direct light). Also the liquid culture of the unconjugated strain was difficult to grow and died after a short time of 3 weeks. The other two strains preserved the normal concentration of antibiotics, but the culture of 7120 *qox*$^-$ (pRStUV3) was bleached after three weeks and had to be transferred to a new plate earlier.

The following combinations of antibiotics were shown to enable growth of the cultures and be selective at the same time:

Table 18: Antibiotics for selection after conjugation

	Gentamycin	**Spectinomycin**	**Neomycin**
7120 *Cox*$^-$ (pRStUV1/2)	15	20	-
7120 *Qox*$^-$ (pRStUV3)	-	20	20
7120 *A1*$^-$ *A2*$^-$ (pRStUV1/2)	12	10	10

Part 2: sugar transporter mutants

Overview:

For the generation of sugar transporter mutants in *Synechocystis* sp. PCC 6803 two vectors had to be constructed:

- pEHUV2 for knocking out *lacF*
- pFDUV2 for knocking out *melB*

The constructed vectors were used to knock out the genes. The vector pALUV12 from an earlier work in this laboratory was used for generating the *gtr*$^{-}$ mutant. Single, double and triple mutants were produced. They were characterised regarding their ability to grow on high amounts of fructose in liquid and on solid medium and their ability to incorporate fructose.

Construction of a knock out vector for *lacF*

Step 1: Cloning *slr1202* into a vector

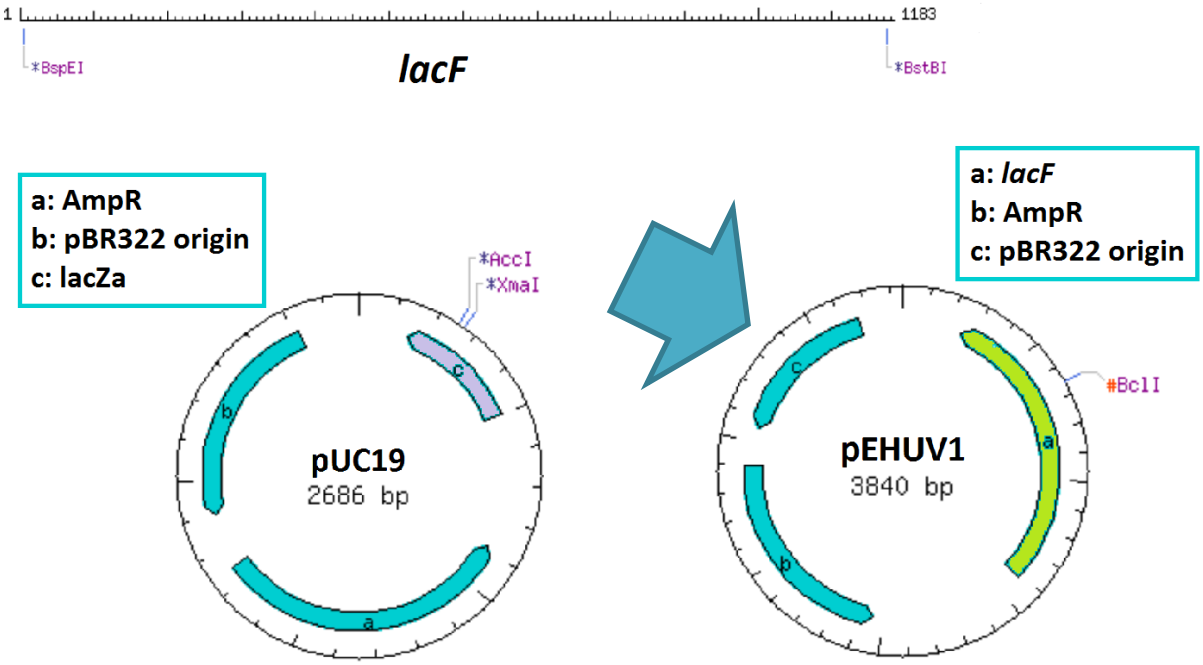


Fig. 13: Construction of pEHUV1

A genomic DNA isolation of *Synechocystis* sp. PCC 6803 was performed and the PCR of the gene *slr120*, was performed, see 'Material and methods, Amplifying DNA with Polymerase Chain Reaction'. The primer contained sites for BstEI and BspBI and PCR product was digested with these two restriction enzymes. The pUC19-vector was isolated and digested

with XmaI and AccI, which produce ends compatible to BstEI and BspBI respectively. The 1183 bp fragment of *lacF* and the double digested 2670 bp long fragment of pUC19 were purified by an agarose gel and ligated at room temperature for two hours. The plasmid was transformed into *E. coli* DH5α via heat shock, see 'material and methods, Transferring DNA', and a blue-white-screening was performed. The transformants were selected with 50 µg/mL ampicillin. Some of the white colonies were used to inoculate each a 3 mL liquid culture of LB and a plasmid preparation was performed. Digestions of the plasmid with different restriction enzymes proved the successful construction of the plasmid pEHUV1, see Fig. 13.

Step 2: Interrupting *slr1202* with an antibiotic cassette

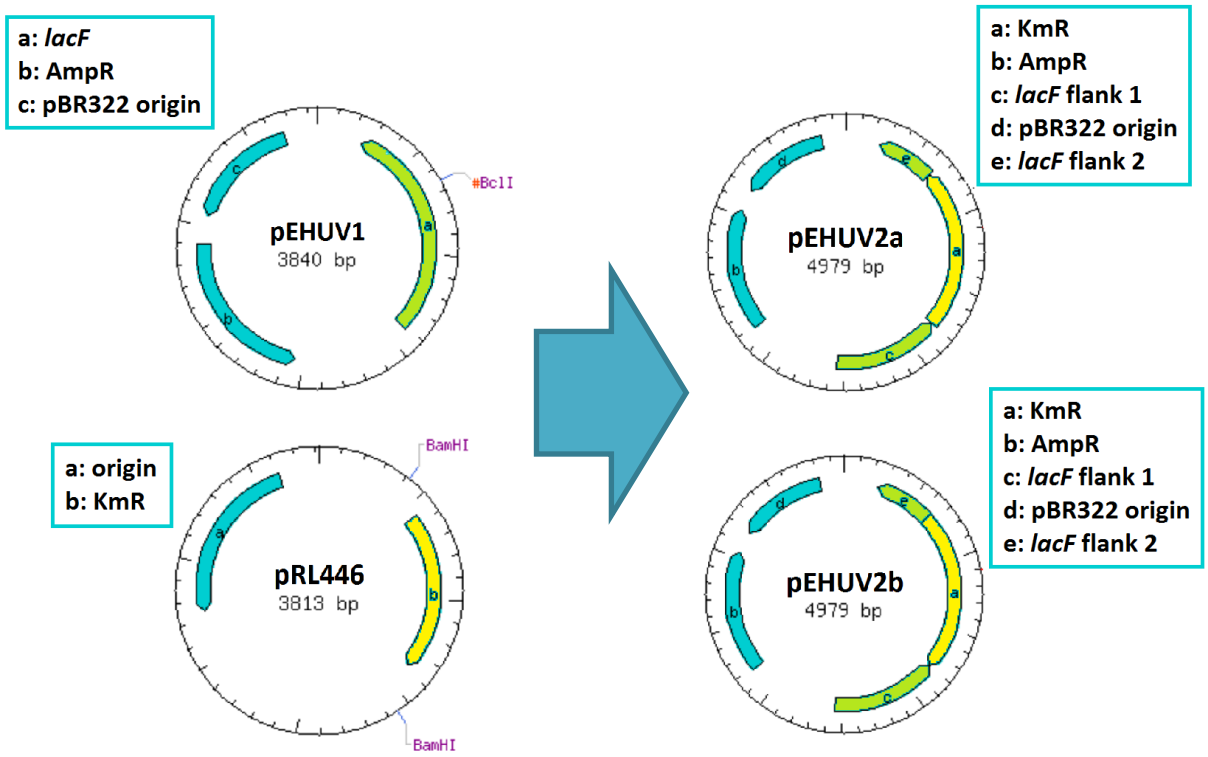


Fig. 14: Construction of pEHUV2a and b

For using the dam-methylation sensitive BclI site for inserting the Km^R cassette, the plasmid pEFUV1 was transformed into *E. coli* GM48 by electroporation, a strain which is *dam*$^-$ and *dcm*$^-$. The preparation of the cells and the electroporation was performed as described in 'Material and methods, Transformation of *Escherichia coli*'. The transformants were selected with 50 µg/mL ampicillin on LB agar plates. A liquid culture was inoculated and plasmid isolation was done. The methylation free pEFUV1 was digested with Bcl1 and the 3840 bp long fragment was purified on an agarose gel.

A plasmid preparation of a liquid culture of HB101 (pRL446) was performed. This plasmid contains a Km^R cassette, which was cut out by digestion with BamHI. The *ca*. 1.1 kbp long fragment was purified on an agarose gel and mixed with the BclI-digested pEFUV1 for ligation, see 'Material and methods, Ligation'. The reaction was performed at room temperature for two hours. The DNA was transformed by heat shock into *E. coli* DH5α. The

cells were streaked out on an LB agar plate containing 50 µg/mL kanamycin. Some colonies were used to inoculate each a liquid culture of LB and plasmid isolations were performed. The isolated plasmids were digested with restriction enzymes and an agarose gel showed that the constructions of pEFUV2a and b were successful: The Km^R was inserted parallel to the direction of the gene for pEHUV2a and antiparallel for pEHUV2b, as shown in Fig. 14.

Construction of a knock out vector for *melB*

Step 1: Cloning *sll1374* into a vector

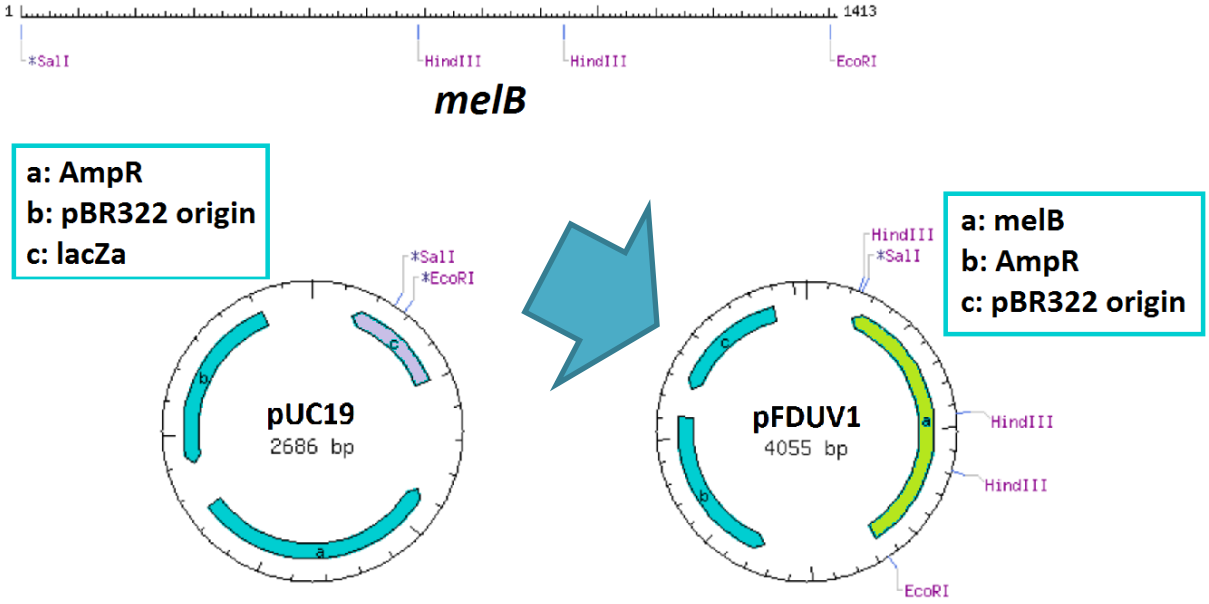


Fig. 15: Construction of pFDUV1

A genomic DNA isolation of *Synechocystis* sp. PCC 6803 and a PCR of the gene *sll1374* was performed. The primers contained sites for SalI and EcoRI, as did the vector pUC19. The 1396 bp long PCR product and the pUC19 vector were each digested with SalI and EcoRI.

The fragments were purified by an agarose gel and ligated at room temperature for two hours. The ligation mix was used for a transformation into *E. coli* DH5α by the heat shock method. Then a blue-white-screening was performed. The plates contained 50 µg/mL ampicillin. Some of the white colonies were used to inoculate each a liquid culture of LB and plasmid preparations were performed.

Digestions of the plasmid with different restriction enzymes proved the successful construction of the plasmid pFDUV1, as shown in Fig. 15.

Step 2: Interrupting *sll1374* with an antibiotic cassette

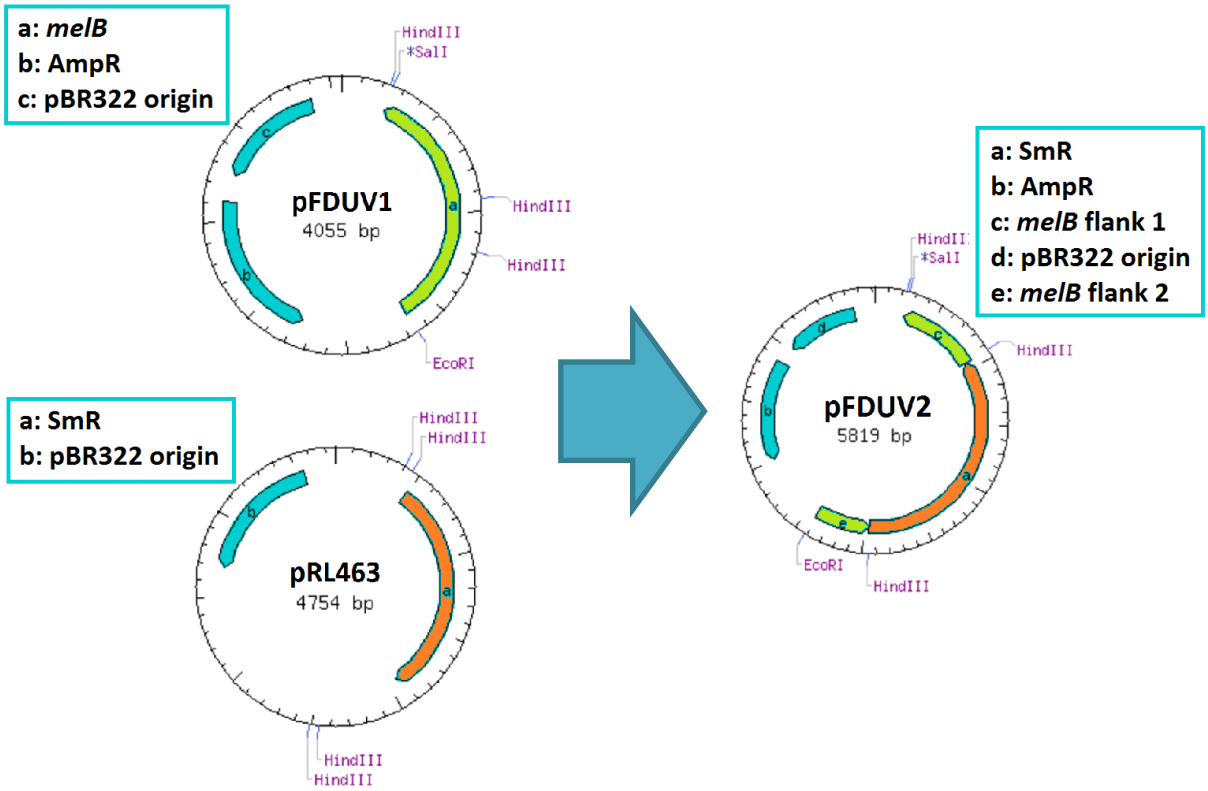
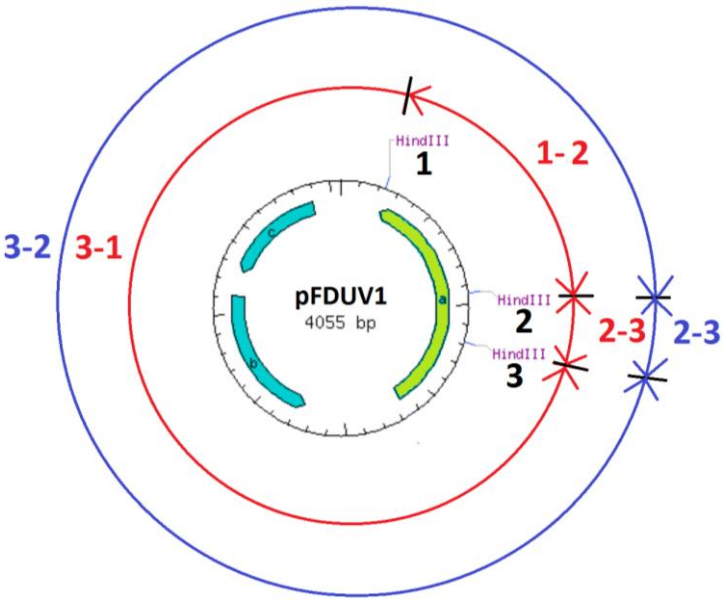


Fig. 16: Construction of pFDUV2

The plasmid pRL463 contains a 2 kbp long Sm^R/Sp^R cassette which was cut out by digestion with the enzyme HindIII. The plasmid pFDUV1 contains 3 sites for HindIII, two in the middle of the *melB* gene and one outside of the *melB* gene in the multiple cloning site of pUC19 as can be seen in Fig. 16.

An incomplete digestion of pFDUV1 with HindIII was performed: the reaction was set up at room temperature instead of 37°C as recommended for a complete digestion and every minute the reaction was stopped for one sample by heating this sample to 80°C for 20 minutes. The result is a mixture of different fragments, as can be seen in Fig. 17, which were separated on an agarose gel.

The highest yield of the fragment HindIII 3–2 could be achieved after incubation for 4 to 5 minutes. The 3805 bp long HindIII 3–2 fragment was extracted from the gel and a ligation with the Sm^R/Sp^R fragment was set up as before. The subsequent heat shock transformation of the mix into *E. coli* DH5α led to several transformants on an LB agar plate containing 50 µg/mL streptomycin. A plasmid preparation of some of the colonies and a digest by different restriction enzymes showed that one colony of transformed cells carried the expected plasmid: pFDUV2, only the version with parallel gene and Sm^R could be obtained, as shown in Fig. 16.



possible fragments	base pairs (bp)
HindIII 1-2	708
HindIII 2-1	3347
HindIII 2-3	250
HindIII 3-2	3805
HindIII 1-3	958
HindIII 3-1	3097

Fig. 17: HindIII fragments of pFDUV1

Transformation of pALUV12, pEHUV2 and pFDUV2 into *Synechocystis* sp. PCC 6803

The following transformations were made:

Table 19: Sugar transporter mutants

strain	genotype	plasmid	new strain	new genotype
6803	wt	pALUV12	6803 *gtr*$^-$	*gtr*::Em^R
6803	wt	pEHUV2a	6803 *lacF*$^-$	*lacF*::Km^R
6803	wt	pEHUV2b	6803 *lacF*$^-$	*lacF*::Km^R
6803	wt	pFDUV2	6803 *melB*$^-$	*melB*::Sm^R
6803 GS1	*gtr*::Km^R	pFDUV2	6803 *GB*$^-$	*gtr*::Em^R, *melB*::Sm^R
6803 *gtr*$^-$	*gtr*::Em^R	pEHUV2a	6803 *GF*$^-$	*gtr*::Em^R, *lacF*::Km^R
6803 *gtr*$^-$	*gtr*::Em^R	pEHUV2b	6803 *GF*$^-$	*gtr*::Em^R, *lacF*::Km^R
6803 *lacF*$^-$	*lacF*::Km^R	pFDUV2	6803 *BF*	*lacF*::Km^R, *melB*::Sm^R
6803 *GF*$^-$	*gtr*::Em^R, *lacF*::Km^R	pFDUV2	6803 *3f*	*gtr*::Em^R, *lacF*::Km^R, *melB*::Sm^R

The transformations were made in three steps:

1. Producing single mutants
2. Producing double mutants
3. Producing the triple mutant

First a *Synechocystis* sp. PCC 6803 wildtype liquid culture of 50 mL was inoculated and grown at 32°C and under permanent light for 3 weeks (OD_{730} = 4-6).

Plasmid preparations were made from *E. coli* DH5α (pALUV12), DH5α (pEHUV2a), DH5α (pEHUV2b) and DH5α (pFDUV2). The plasmid solutions were digested with RNase and purified via an agarose gel.

The transformation of the plasmids was performed as described in ‘material and methods, Transformation into *Synechocystis* sp. PCC 6803’. After two weeks of incubation four colonies of each mutant were used to inoculate each a 50 mL liquid culture of BG11TS + antibiotics, see table 20.

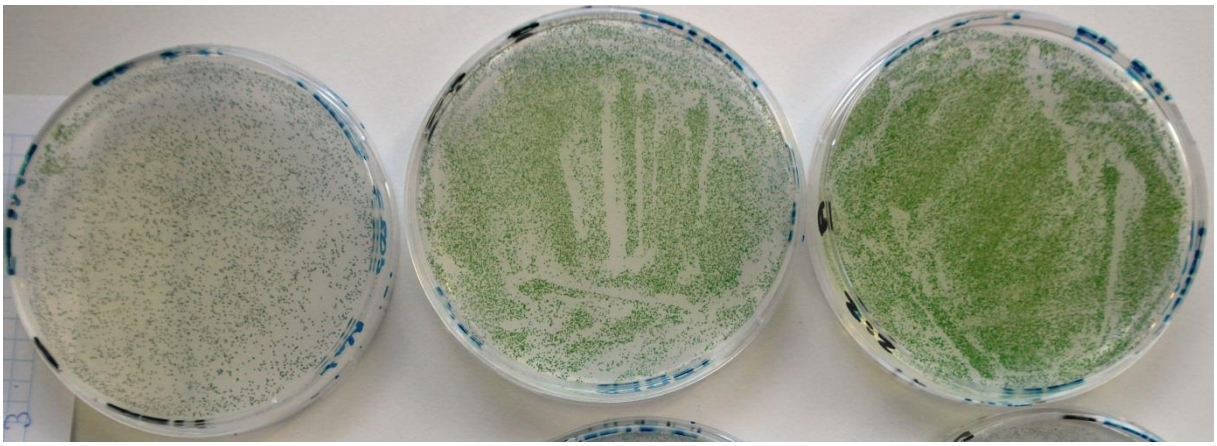


Fig. 18: selective plates of transformation into 6803 wt after 2 weeks with 0.5 µL, 1 µL and 2 µL of pEFUV2 isolate

The liquid cultures were grown for two to three weeks at 32°C, 60 % air humidity, permanent shaking with 130 rpm and in permanent light.

In contrast to the wildtype, none of the colony PCRs from the mutants led to a PCR product. Therefore a preparation of genomic DNA was performed as described in ‘Material and methods, Preparation of genomic DNA from Cyanobacteria’ and later using the xanthogenate method as described in ‘Material and methods, Preparation of genomic DNA from *Synechocystis* sp. PCC 6803 via Xanthogenate method’. A PCR was performed for each mutant to prove homozygocity.

When the cultures proved to be homozygous they were used for further transformations, first for producing the double mutants and after that the triple mutant, as can be seen in table 17. The process was the same as for the single mutants.

The cultures of 6803 *gtr*$^{-}$ were homozygous after the first two weeks. The cultures of 6803 *lacF*$^{-}$ were grown for 4 weeks until they became homozygous. The mutants that were altered in the *melB* gene, single, double and triple mutants, did not become homozygous within 12 weeks.

Fig. 19 shows the agarose gels of PCR products of genomic DNA preparations of mutants and wildtype:

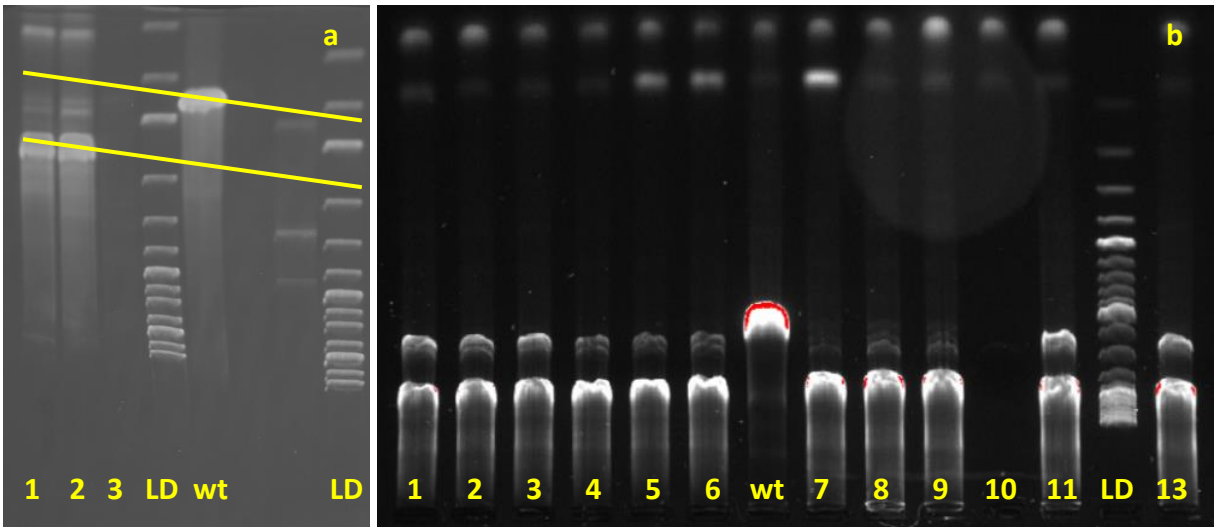


Fig. 19: PCR products of a test on homozygoty; LD = GeneRuler 1 kb ladder ready to use mix; wt = wildtype; 19a: 1, 2, 3 = samples of the cultures PCC 6803 *gtr*⁻ 1, 2 and 3; 1 and 2 are homozygous; 19b: 1 – 13 = samples of the cultures PCC 6803 *melB*⁻ 1 to 13, all samples are not homozygous;

The gel shown in figure 19a did not run homogeneously, see yellow lines. Therefore no wildtype PCR products were detected in samples 1 and 2. Sample 3 was disregarded because it did not yield a PCR product at all. The gel shows the homozygous genes of PCC 6803 *gtr*⁻ (2360 base pairs) in comparison to the wildtype gene of the gtr carrier (863 base pairs).

Figure 19b shows the non-homozygous genes of various PCC 6803 *melB*⁻ mutants. The 1396 base pairs long wildtype gene of *melB* can be seen in every sample. The knocked out gene is 3166 base pairs long.

Only homozygous cultures were used for further experiments. As the *melB*⁻ mutants did not become homozygous in time, they were not used for sugar uptake studies and remain object of further characterisation in other works.

The following concentrations of antibiotics were chosen for the selection:

Table 2012: Antibiotics for selection after transformation

new strain	new genotype	Antibiotics in µg/mL		
		Erythromycin	Streptomycin	Kanamycin
6803 *gtr*$^-$	*gtr*::Em^R	12	-	-
6803 *lacF*$^-$	*lacF*::Km^R	-	-	20
6803 *lacF*$^-$	*lacF*::Km^R	-	-	20
6803 *melB*$^-$	*melB*::Sm^R	-	20	-
6803 *GB*$^-$	*gtr*::Em^R, *melB*::Sm^R	10	20	-
6803 *GF*$^-$	*gtr*::Em^R, *lacF*::Km^R	10	-	20
6803 *GF*$^-$	*gtr*::Em^R, *lacF*::Km^R	10	-	20
6803 *BF*$^-$	*lacF*::Km^R, *melB*::Sm^R	-	20	20
6803 *3f*	*gtr*::Em^R, *lacF*::Km^R, *melB*::Sm^R	10	15	15

Experiments in liquid cultures

Conditions and observations

To test the effect of knocking out the sugar carrier *lacF*, different combinations of different sugars were chosen for the strains 6803 wt, 6803 *gtr*$^-$, 6803 *lacF*$^-$ and 6803 *GF*$^-$:

Table 213: Set up for experiments in liquid cultures

medium	additive/s	growth mode
BG11-TS + antibiotics	-; light	photo-litho-autotrophic
BG11-TS + antibiotics +	50 mM fructose; light	mixotrophic
BG11-TS + antibiotics +	100 mM fructose; light	mixotrophic
BG11-TS + antibiotics +	200 mM fructose; light	mixotrophic
BG11-TS + antibiotics +	50 mM glucose; light	mixotrophic
BG11-TS + antibiotics +	100 mM glucose; light	mixotrophic
BG11-TS + antibiotics +	200 mM glucose; light	mixotrophic
BG11-TS + antibiotics +	10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	50 mM fructose + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	100 mM fructose + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	200 mM fructose + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	50 mM glucose + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	100 mM glucose + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	200 mM glucose + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	50 mM sorbitol + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	200 mM sorbitol + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	50 mM frc. + 150 mM sorb. + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	50 mM glc + 150 mM sorb. + 10 µM DCMU; light	heterotrophic
BG11-TS + antibiotics +	-; darkness	heterotrophic
BG11-TS + antibiotics +	200 mM fructose; darkness	heterotrophic
BG11-TS + antibiotics +	200 mM fructose + 10 µM DCMU; darkness	heterotrophic

All media were prepared according to the table above:

- fructose stock: 2 M in BG11-TS
- glucose stock: 2 M in BG11-TS
- sorbitol stock: 2 M in BG11-TS
- DCMU stock: 20 mM in ethanol

The observation started at an OD_{730} of 0.2 and was continued for four weeks. The liquid cultures were grown at 32°C, 60 % air humidity and under permanent light. Every two to three days the OD_{730} was determined with a spectrophotometer in a semi-micro cuvette, see 'Material and methods, Spectrometry'. 1 mL of each sample was taken for measurement and when the OD_{730} reached 1 or more, the sample was diluted with BG11-TS.

Change of colours in the cultures

An interesting change of colour occurred in the fructose containing cultures of 6803 *GF*¯. The density of the cultures increased with the amount of fructose added and proportionally the supernatant turned darker, as can be seen in Fig. 20.

Fig. 20: Cultures of 6803 *GF*¯ with increasing fructose concentration from left to right after 2 weeks: 0, 10, 50, 100 and 200 mM fructose were added;

A four week old culture of 6803 *GF*¯ was transferred into a 50 mL Falcon tube and centrifuged at room temperature for 10 minutes at 6.000 rpm. The supernatant turned out to be red-brownish dark, whereas the cell pellet was green as usual for this strain. 5 mL of the supernatant were centrifuged in a speed vac with a mass filter of 4 kDa to find out whether the colour originated from a protein or from a smaller molecule dissolved in the supernatant. The colour of the filtrate did not change, but also the protein fraction was coloured dark red-brown.

A scan with an UV-Vis spectrometer from 400 to 800 nm was performed but without any significant peaks that would reveal the reason for the colour. The spectrum did not change significantly when ascorbic acid was added as reduction agent, but the colour turned more

into orange, as can be seen in Fig. 21. The colour may originate from more than one species in the medium.

Fig. 21: left: Comparison of two cultures 6803 *GF*⁻ that were grown for four weeks, the left one with 200 mM fructose and the right one under photo-litho-autotrophic conditions; middle: the supernatant of a three week old centrifuged culture of 6803 *GF* with 200 mM fructose; right: changed colour of the supernatant after ascorbic acid was added;

The absorption by the darker supernatant at 730 nm was negligible in comparison to the high OD_{730} of the culture. When the supernatant turned darker, the cultures were centrifuged and fresh BG11-TS was added prior to the measuring of the OD_{730}.

Another observation revealed that the cultures of the *Synechocystis* sp. PCC 6803 turned blue within short time (several hours) when the concentration of fructose was increased to 500 mM. Normally dying cultures bleach out and the supernatant is without colour. When very high amounts of fructose are added to the medium, the cells seem to lyse and release blue coloured phycocyanin.

Growth rates in liquid cultures in different growth conditions in the light

The wildtype (wt) control

By following the change of the OD_{730} over weeks, growth graphs could be obtained for PCC 6803 wt and the mutants PCC 6803 *gtr*⁻ and PCC 6803 *GF*⁻. As expected the PCC 6803 wt died very soon after adding the fructose and grew when glucose was added, as can be seen in Fig. 22.

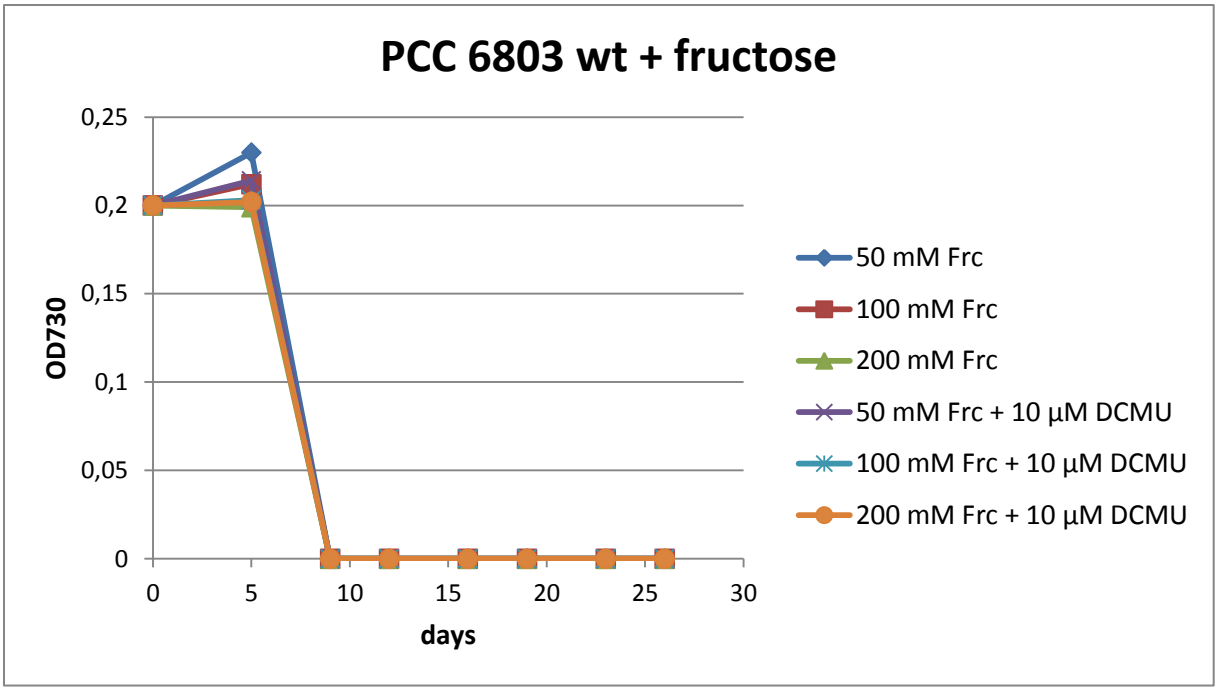


Fig. 22: Fructose has an antibiotic effect on PCC 6803 wt

Fig. 22 shows that adding fructose in any of the used concentrations led to the death of the cultures within several days and no growth could be observed.

Sorbitol is a polyalcohol that cannot be used for heterotrophic or mixotrophic growth.

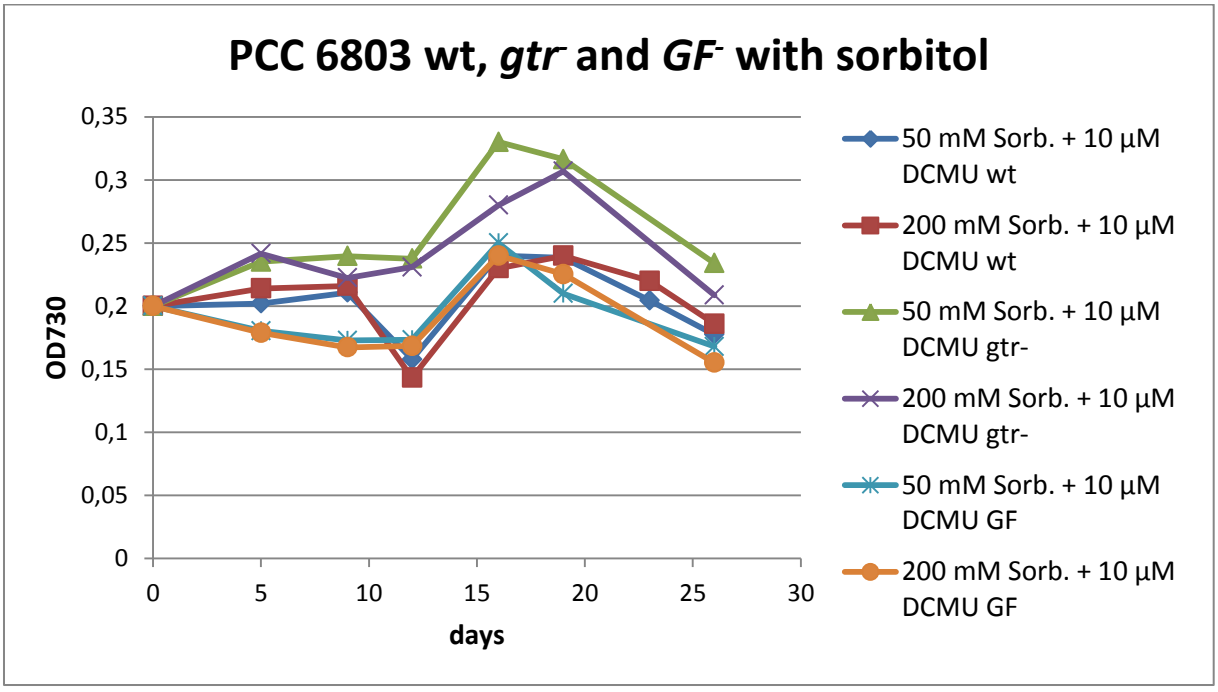


Fig. 23: Development of the cell density when sorbitol and DCMU are added

Cultures of 6803 wt with sorbitol and DCMU could not increase their optical density and died slowly due to the lack of an energy source. As can be seen in Fig 23 the cultures of the

mutants also did not grow on sorbitol. The differences in the growth rates of the different cultures were not significant.

As can be seen in Fig. 24, PCC 6803 wt can grow on glucose. The heterotrophic and mixotrophic growth is similar to that of photo-litho-autotrophic conditions.

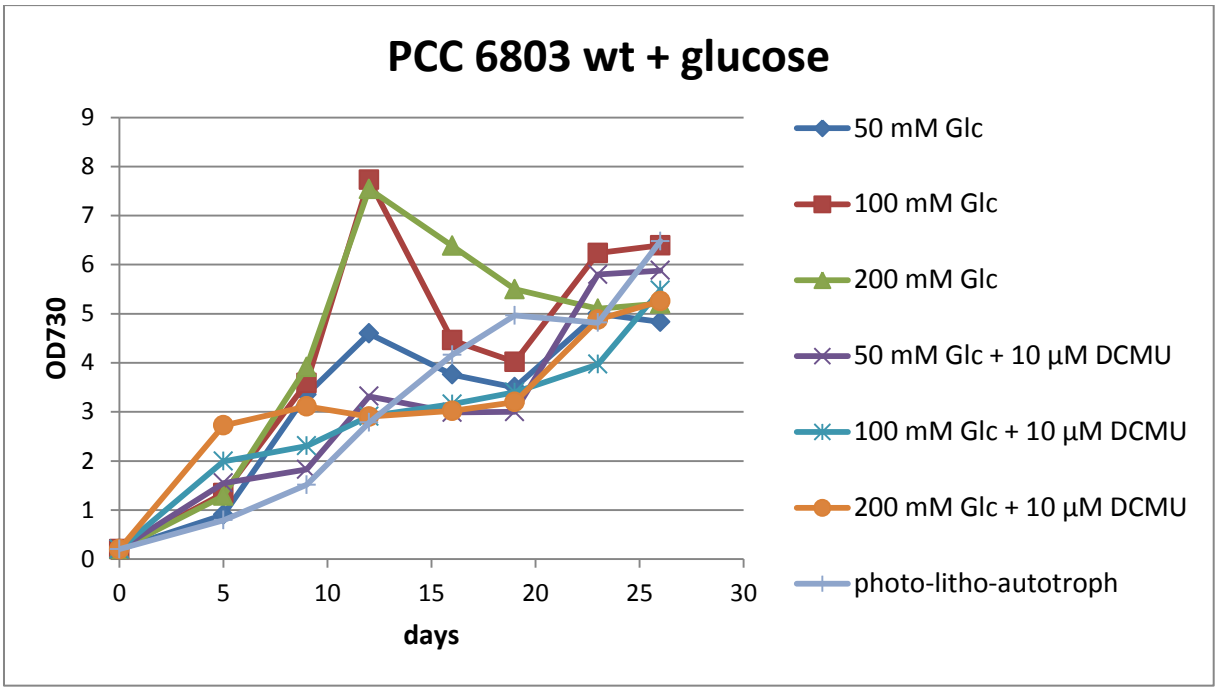


Fig. 24: Growth of PCC 6803 wt on different concentrations of glucose, with and without DCMU

Fig. 24 shows a peak in the OD_{730} after 12 days for the cultures grown on 100 mM and 200 mM under mixotrophic conditions. The reason for the peaks and the drop of cell density after these 12 days are not yet clear.

Comparison of the mutants PCC 6803 *gtr*$^-$ and *GF*$^-$

The *gtr*$^-$ mutant responded to glucose as expected: the glucose carrier was knocked out, therefore there was no increase of the cell density by growing the bacteria heterotrophically on glucose + DCMU.

Under mixotrophic conditions the cell number increased, as can be seen in Fig. 25. The mixotrophic growth of PCC 6803 *gtr*$^-$ was equivalent (regarding the cell density) to the photo-litho-autotrophic growth of the mutant.

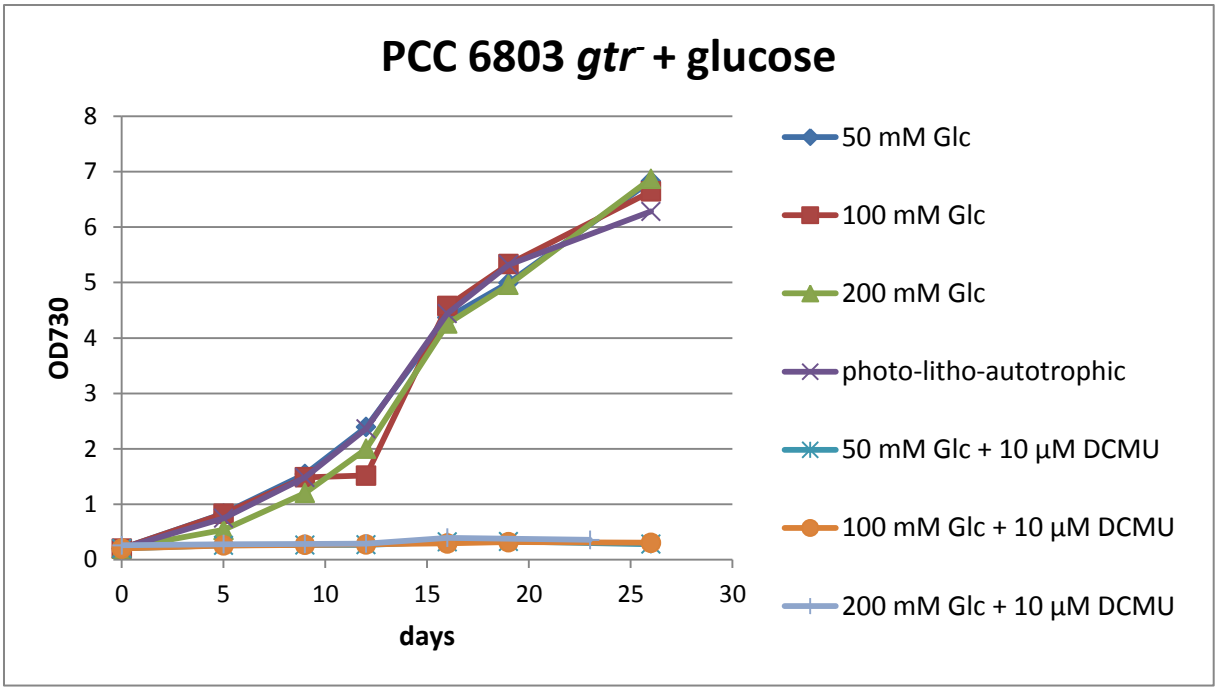


Fig. 25: Growth of the mutant PCC 6803 *gtr*⁻ with glucose

As shown in Fig. 26, the PCC 6803 *GF*⁻ mutant follows the same pattern regarding the growth on glucose as the PCC 6803 *gtr*⁻ mutant. Both mutants lack the *gtr* carrier and it has been shown before, that the *gtr*⁻ mutant cannot incorporate glucose anymore once the carrier is knocked out. The data show that this also applies to the PCC 6803 *GF*⁻ mutant.

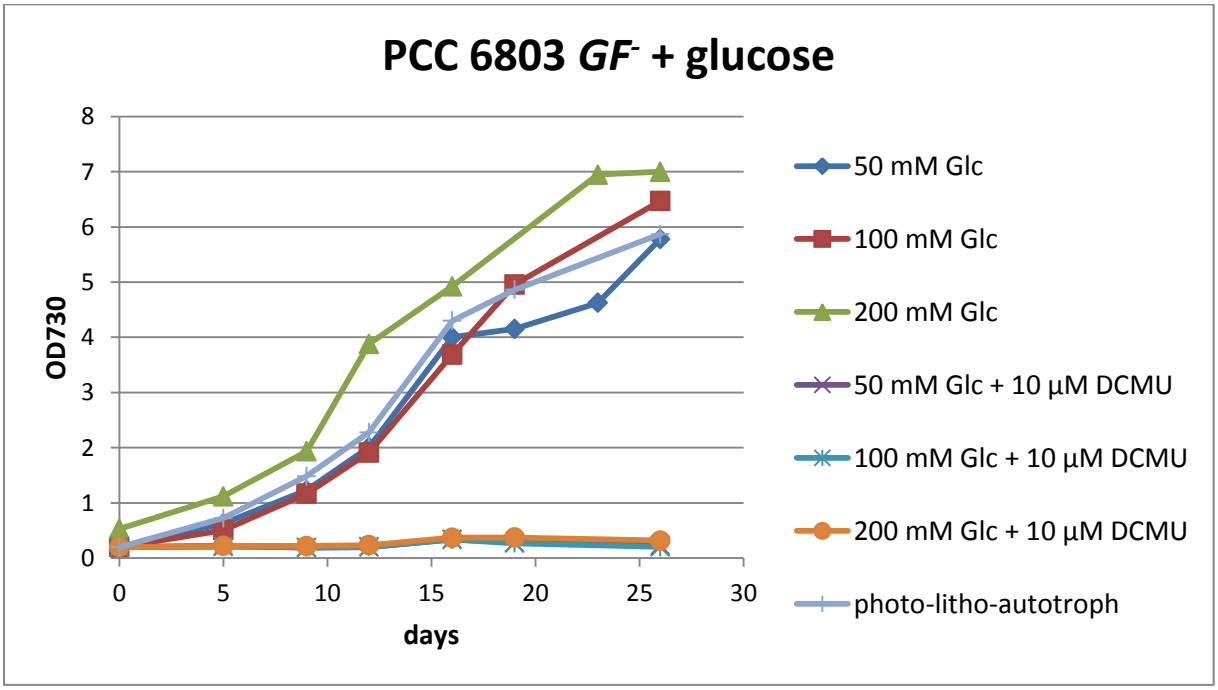


Fig. 26: Growth of PCC 6803 *GF*⁻ on different concentrations of glucose, with and without glucose

Much is known about the uptake mechanism and the metabolism of glucose in *Synechocystis* sp. PCC 6803. It was shown for *Synechocystis* that fructose has an affinity towards the glucose permease as well, but that a toxic metabolite leads to the death of the cell (Flores & Schmetterer, 1986). Once the carrier is knocked out, the PCC 6803 *gtr*⁻ mutant can grow on fructose. The data shown in Fig. 27 suggest, that PCC 6803 *gtr*⁻ is not only resistant to fructose but can also grow heterotrophically on high amounts of this sugar.

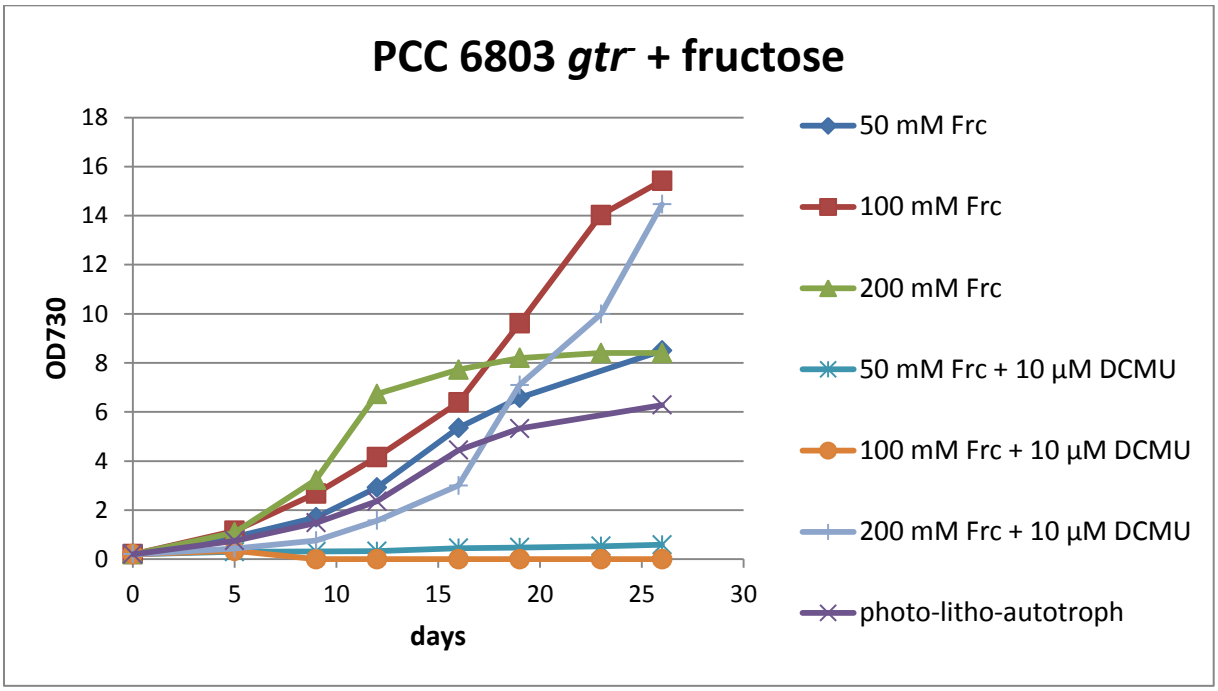


Fig. 27: PCC 6803 *gtr*⁻ growing on fructose with and without DCMU

The experiments showed that the growth of PCC 6803 *gtr*⁻ on fructose under mixotrophic conditions is quite enhanced in comparison to the photo-litho-autotrophic mode. The strongest increase was reached for the heterotrophically grown cultures with 200 mM fructose and the mixotrophically grown cultures with 100 mM fructose.

Interestingly the cultures with DCMU and fructose lower than 200 mM, namely 50 mM and 100 mM, did not show a much enhanced growth and grew more slowly than the photo-litho-autotrophic grown culture. A similar behaviour could be observed in the cultures of PCC 6803 *GF*⁻, as shown in Fig. 28. This was quite interesting because the cultures of PCC 6803 *GF*⁻, in which a second potential sugar carrier is knocked out, were not only not inhibited in their ability to take up fructose, but even enhanced in their growth compared to the *gtr*⁻ mutants and the cultures that grew photo-litho-autotrophically.

Especially the mixotrophically grown cultures of PCC 6803 *gtr*⁻ and *GF*⁻ reached fast unusually high cell densities. The optical densities of PCC 6803 *GF*⁻ mutants have always been higher than those of PCC 6803 *gtr*⁻ mutants.

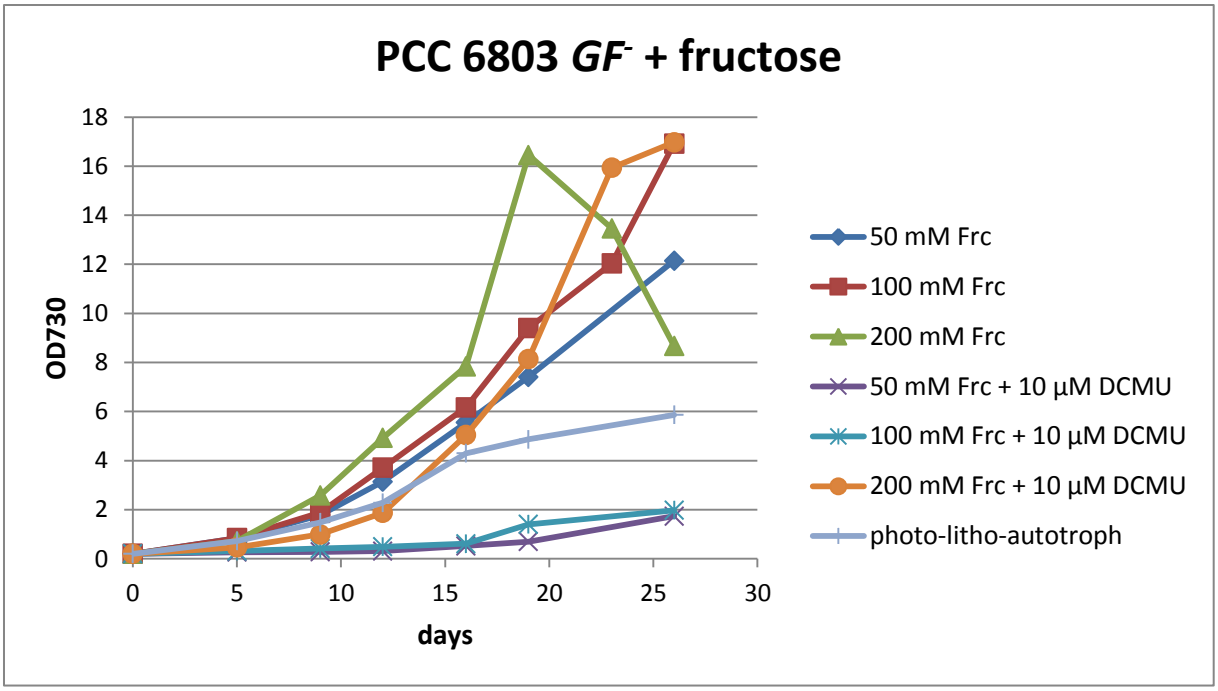


Fig. 28: The growth of PCC 6803 *GF*⁻ on fructose, with and without DCMU

While the mixotrophic cultures of PCC 6803 *gtr*⁻ containing 200 mM fructose did not grow as fast as the ones with 100 mM fructose and 200 mM fructose + DCMU, the optical density of the equivalent cultures of PCC 6803 *GF*⁻ exhibited a faster growth than every other culture tested. After about 18 to 22 days the cell density dropped rapidly.

As mentioned before, the supernatant of fructose-containing cultures turned darker as the OD_{730} increased above *circa* 10. The absorption by the darker supernatant at 730 nm was negligible in comparison to the high OD_{730} of the culture. Nevertheless the cultures with dark supernatants were centrifuged and fresh BG11-TS was added prior to the measuring of the OD_{730}.

An OD_{730} of 18 was the highest OD for PCC 6803 *GF*⁻ and after the cultures reached this density they died. The cultures of 6803 *GF*⁻ with 200 mM fructose + DCMU started more slowly than the mixotrophic equivalent but did also accelerate in growth soon and also reached very high densities, as did the mixotrophic cultures with lower concentrations of fructose.

These cultures merely needed more time than the mixotrophic grown culture with 200 mM fructose. This supports the assumption that the fast growth is caused by the high concentration of fructose. Fig. 29 shows that the sample of PCC 6803 *gtr*⁻ with 200 mM fructose reached a plateau after a while, whereas the organo-heterotrophic culture of this mutant grew slower in the beginning, but finally surpassed its mixotrophically grown counterpart.

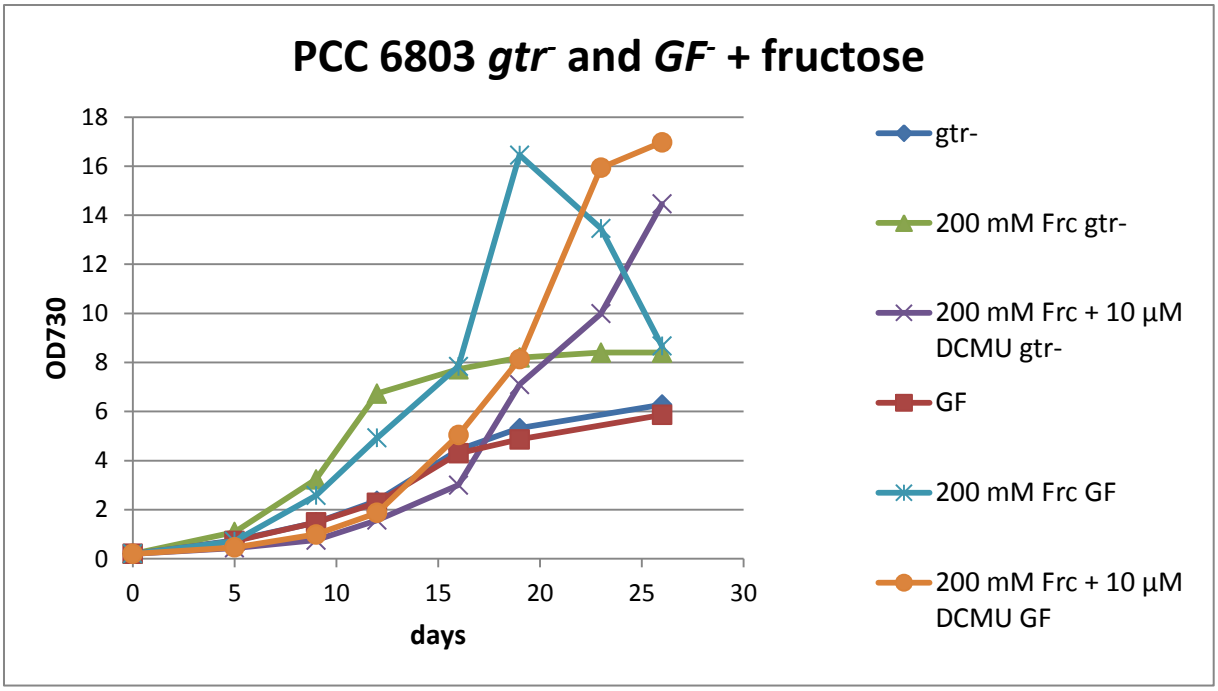


Fig. 29: Optical densities of the mutants PCC 6803 *gtr*⁻ and *GF*⁻ with 200 mM fructose in comparison to the photo-litho-autotrophically grown cultures

The growth of the cultures was almost proportional to the amount of fructose that was used. In the first screening for the assay in liquid cultures the concentration of 10 mM fructose was used. The growth strongly correlates to the fructose concentration under heterotrophic conditions as well as under mixotrophic conditions, as can be seen in fig. 30 for heterotrophic growth conditions.

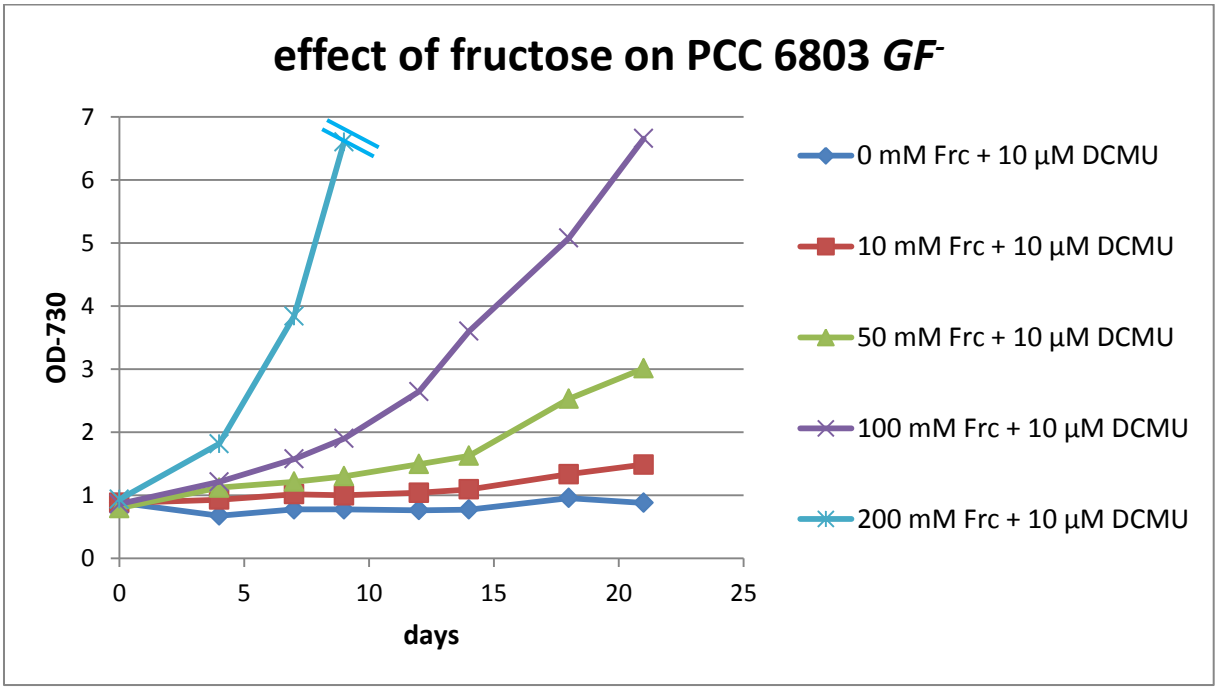


Fig. 30: Effect of fructose on PCC 6803 *GF*⁻

The culture of PCC 6803 *GF*$^-$ with 200 mM fructose + 10 µM DCMU peaks at OD_{730} = 18 and is not shown completely in Fig. 30.

To determine the role of osmotic effects concerning the uptake of fructose, cultures with the same osmolarity but with lower concentrations of fructose were investigated, as can be seen in Fig. 31 and 32. Sorbitol was added to the cultures until a certain osmolarity was reached.

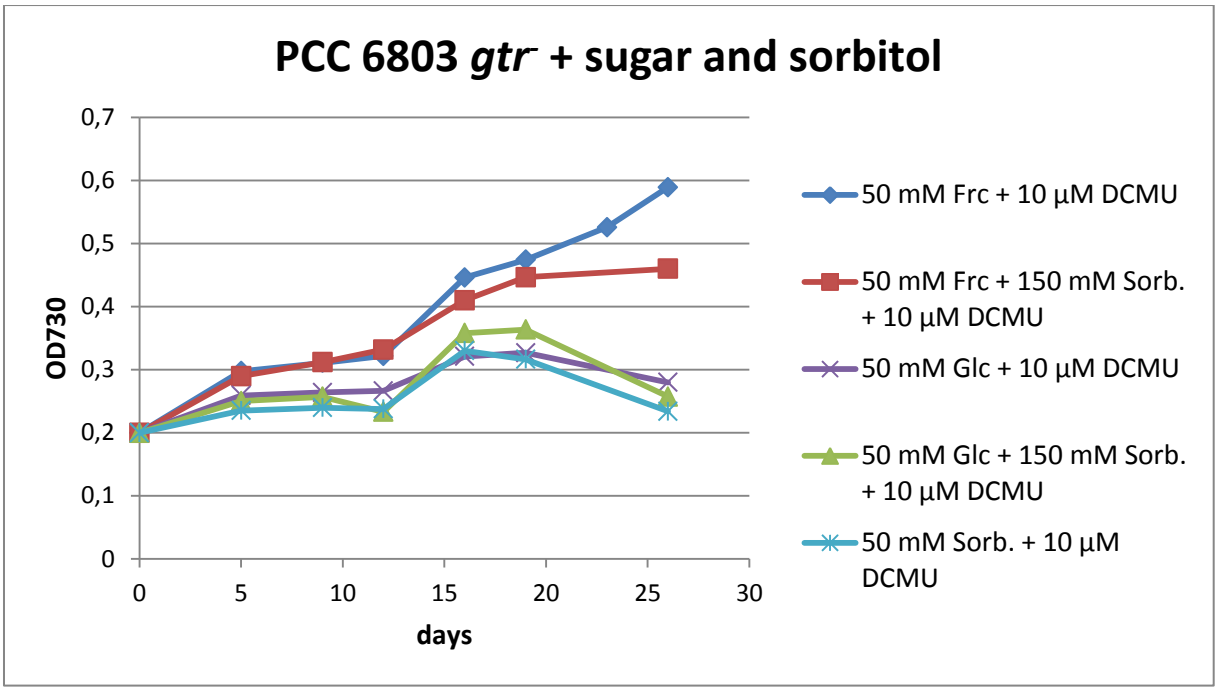


Fig. 31: Comparison of PCC 6803 *gtr*$^-$ + 50 mM sugar + 150 mM sorbitol + DCMU to PCC 6803 *gtr*$^-$ + 50 mM sugar + DCMU

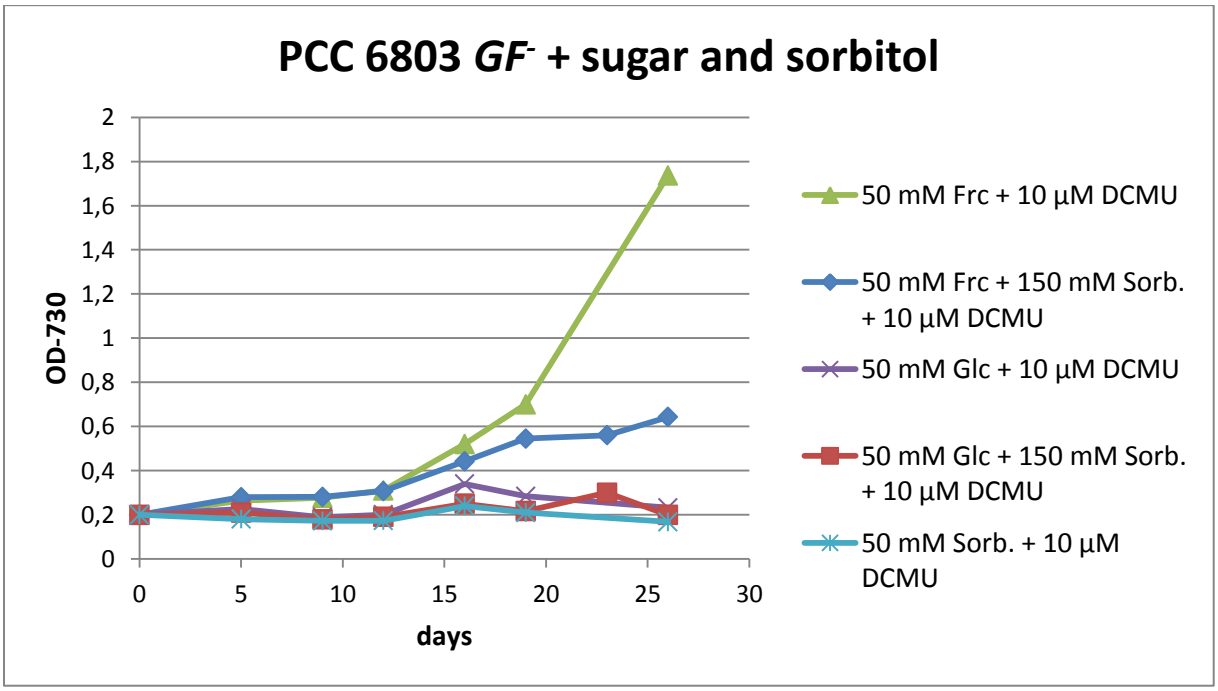


Fig. 32: Comparison of PCC 6803 *GF*$^-$ + 50 mM sugar + 150 mM sorbitol + DCMU to PCC 6803 *GF*$^-$ + 50 mM sugar + DCMU

As can be seen in Fig 31 and Fig. 32 both mutants reacted the same way. As expected, the cultures with 50 mM glucose, 50 mM glucose and 150 mM sorbitol + DCMU and 50 mM sorbitol + DCMU did not grow properly. The cultures with 50 mM fructose and 150 mM sorbitol + DCMU did not show a strongly accelerated growth when compared to cultures with the same osmolarity but without sorbitol. The uptake of fructose or the growth seemed to be impaired by sorbitol. The optical density of these cultures increased slowly and was also higher for PCC 6803 *GF*$^-$ than for the PCC 6803 *gtr*$^-$.

Growth rates in liquid cultures in different growth conditions in the dark

The experiments in the dark showed no significant influence on the growth when adding 200 mM fructose to the medium within three weeks, but the observation time may have been too short to observe any effects.

Fig. 33 shows the graphs for the growth in the dark of the two mutants PCC 6803 *gtr*$^-$ and PCC 6803 *GF*$^-$ with and without 200 mM fructose and with and without DCMU.

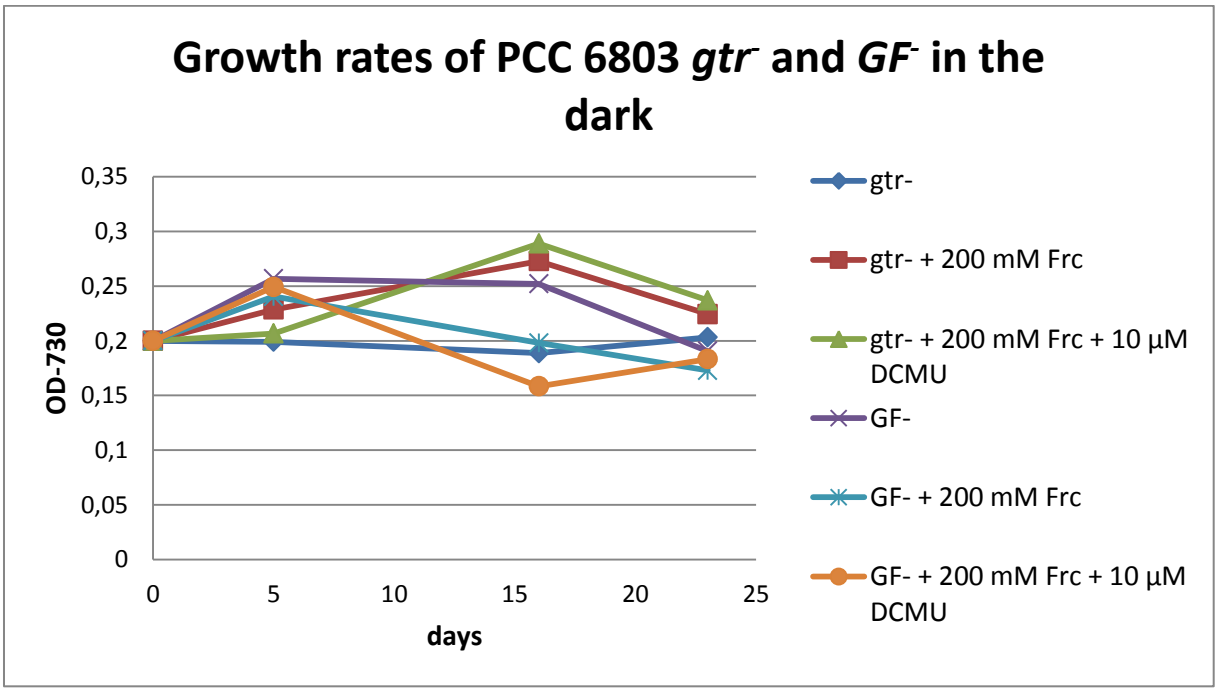


Fig. 33: Growth rates of PCC 6803 *gtr*$^-$ and PCC 6803 *GF*$^-$ in the dark

As can be seen in Fig. 33, there was no significant difference between the growth rates of the two mutants. The OD_{730} did not change significantly in the cultures with 200 mM fructose. DCMU did not influence the optical density as well.

Uptake experiments with ^{14}C-uniformly labelled fructose

The following experiments were performed:

Table 14: Experiments with radioactively labelled fructose

Mutant	concentration of fructose in the medium during measurement	grown on fructose in advance (5 days)	
		yes	no
GS1	200 mM	-	X
	200 mM	200 mM	
	10 mM	200 mM	
	10 mM	10 mM	
GF	200 mM	-	X
	200 mM	200 mM	
	200 mM	10 mM	
	10 mM	200 mM	
	10 mM	10 mM	

The measurements were made with the Perkin Elmer liquid scintillation analyser Tri-Carb 2800 TR.

Fig. 34: radioactively labelled samples in the scintillator ready for measurement

Results of the uptake measurements

The aim of the experiment was to study the influence of the fructose concentration on its uptake and also how pretreatment with fructose changes the uptake behaviour. The pretreated cultures were grown on 10 mM or 200 mM fructose 5 days in advance to the uptake measurements.

As shown in Fig. 35, pretreatment of the liquid culture with 10 mM fructose led to an increased uptake of fructose in PCC 6803 *gtr*⁻ compared to the not pretreated sample.

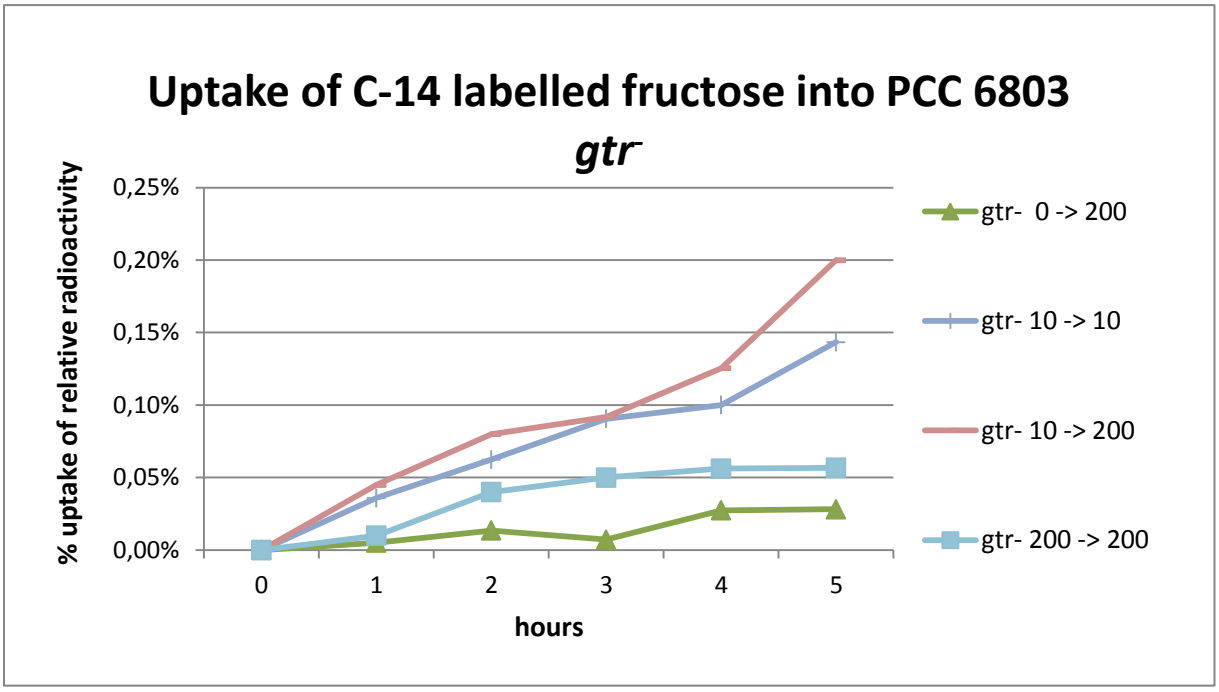


Fig. 35: Relative uptake of radioactively labelled fructose into PCC 6803 *gtr*⁻ under different conditions. Legend: 10 → 200 = grown on 10 mM fructose for 5 days before the uptake experiment → treated with 200 mM during the uptake experiment.

Obviously the uptake of fructose is more enhanced by pretreatment than by a high concentration of fructose during the experiment. All three pretreated cultures showed a higher uptake than the not pretreated one.

The culture that was treated with 200 mM fructose in advance and during the experiment did not respond as significantly as the cultures that were pretreated with 10 mM fructose. Regarding the uptake of radioactively labelled fructose it was revealed to be irrelevant whether the cultures were pretreated with 10 mM fructose and exposed to 200 mM fructose during the experiment, or if it was done *vice versa*.

The same behaviour with one change was observed in PCC 6803 *GF*⁻, as can be seen in Fig. 36.

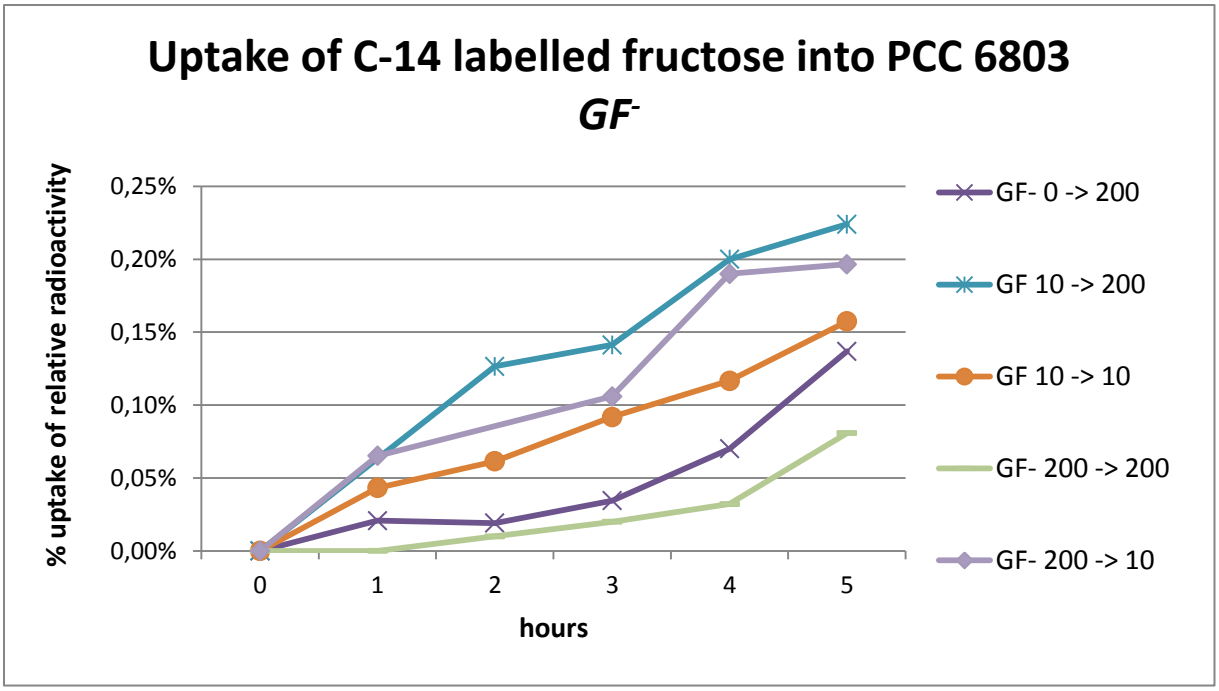


Fig. 36: Relative uptake of radioactively labelled fructose into PCC 6803 *GF*⁻ under different conditions; Legend see Fig. 34

The not pretreated culture took up more radioactivity than the one pretreated with 200 mM fructose. In this mutant the sample pretreated with 200 mM fructose showed the lowest relative uptake of radioactivity.

6803 *GF*⁻ took up slightly more radioactively labelled fructose compared to 6803 *gtr*⁻, as can be seen in Fig. 37.

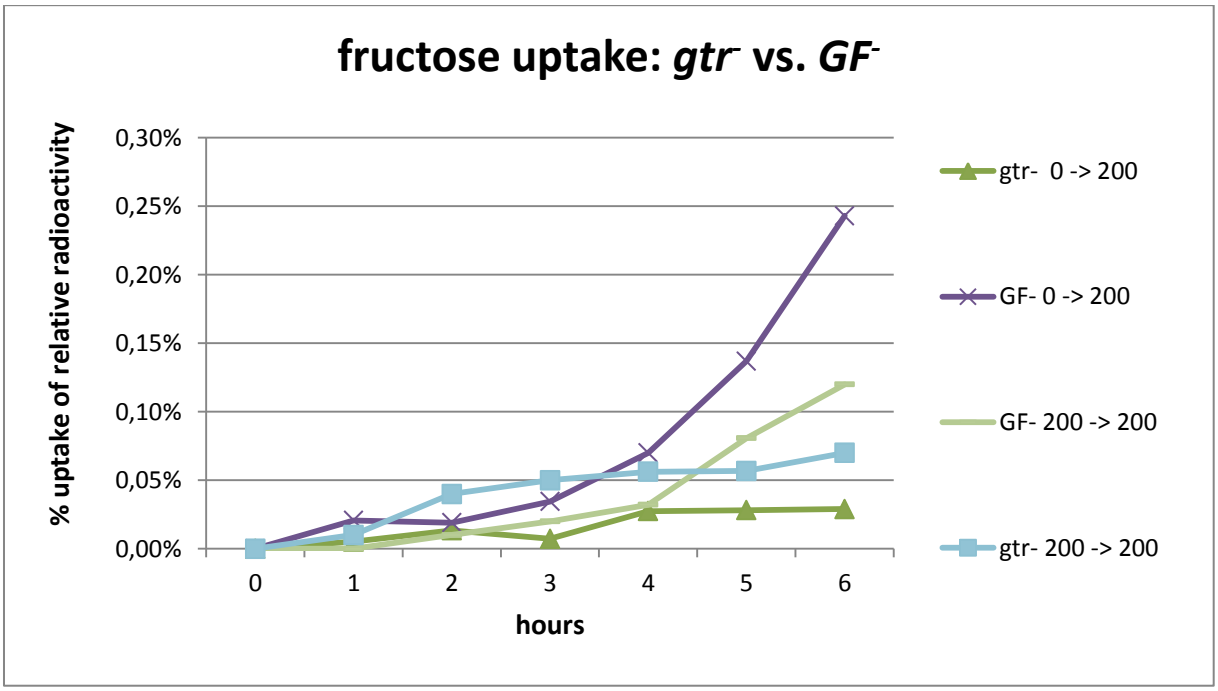


Fig. 37: Comparison between the two mutants PCC 6803 *gtr*⁻ and *GF*⁻ regarding the uptake of radioactively labelled fructose; Legend see Fig. 34;

That means they were able to take up more sugar and this corresponded with the data of the liquid cultures, where the *GF*$^-$ mutant showed a faster growth than the *gtr*$^-$ mutant.

The pretreated cultures of both strains performed similaly, as can be seen in Fig. 38.

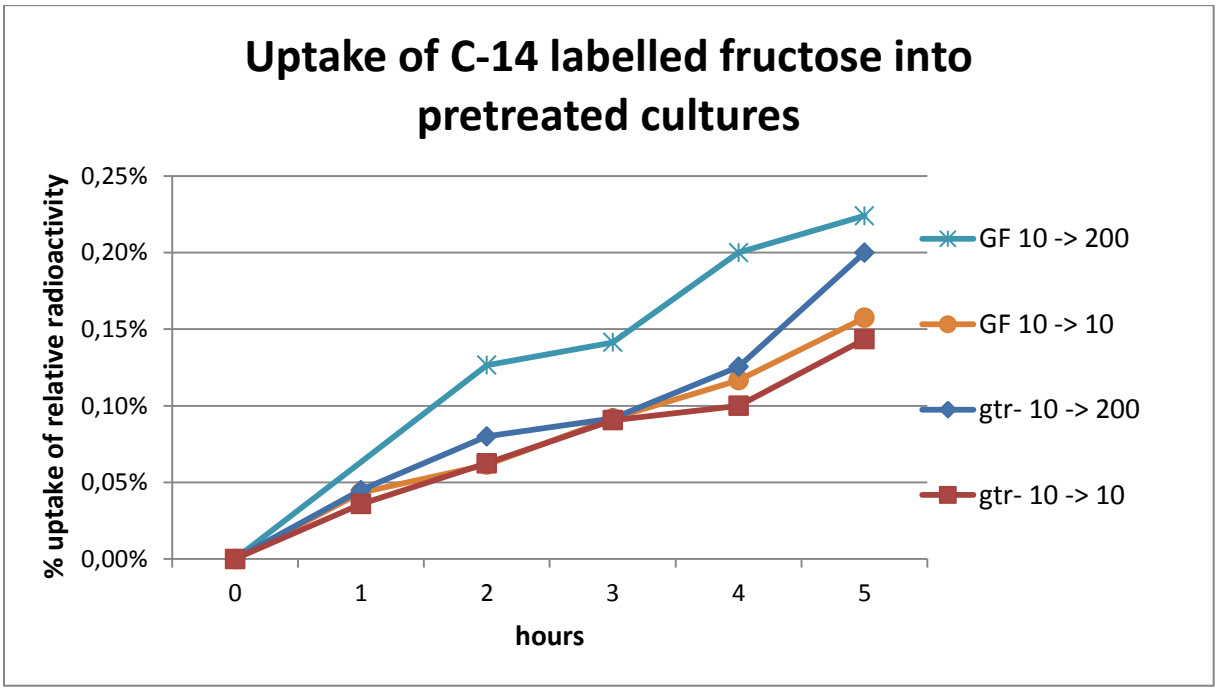


Fig. 38: Comparison of the uptake of pretreated PCC 6803 *gtr*$^-$ and PCC 6803 *GF*$^-$; Legend see Fig 34;

The values for the samples PCC 6803 *gtr*$^-$ 10 → 10 and PCC 6803 *GF*$^-$ 10 → 10 were almost the same, but the values differed when the cultures were pretreated with 200 mM fructose or when higher sugar concentrations were added during the experiment.

Experiments on solid medium

Experiments on BG11-HPA

Whereas PCC 6803 *gtr*$^-$ needs the solid medium BG11-TSA to grow and produce colonies, PCC 6803 *GF*$^-$ is also capable of also growing on the solid medium BG11-HPA. As can be seen in Fig. 39 the colour of the colonies differed on the different media. It was much darker on BG11-HPA than on BG11-TS.

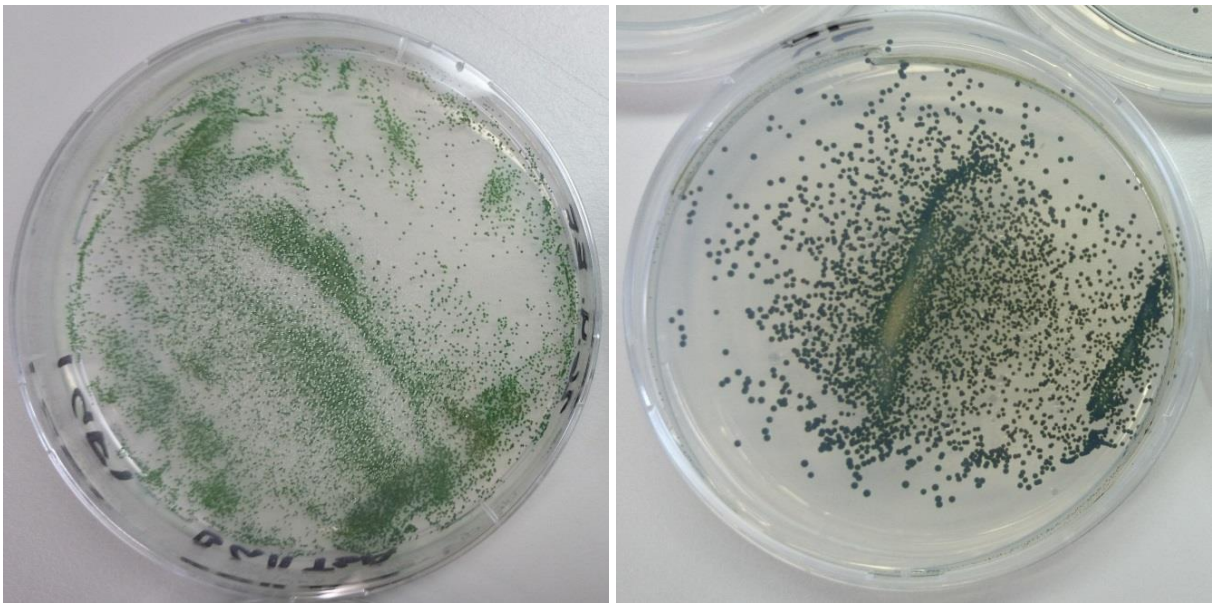


Fig. 39: Two solid media with PCC 6803 *GF*$^-$ incubated for three weeks. Left: BG11-TSA, right: BG11-HPA.

Experiments with fructose

Liquid cultures of PCC 6803 *gtr*$^-$ and PCC 6803 *GF*$^-$ were diluted to an OD_{730} of 0.1. 100 µL (about $1x10^6$ cells) were spread onto petri dishes filled with 25 mL BG11-TSA, half of them containing 100 mM fructose. The plates were incubated for two weeks at 32°C, 80 % air humidity and in permanent light.

On the plates with only BG11-TSA colonies had grown, but the ones with 100 mM fructose seemed to be empty. After further incubation for another two weeks, small colonies could be observed on the plates with 100 mM fructose, as can be seen in Fig. 40 for PCC 6803 *gtr*$^-$ and in Fig. 41 for PCC 6803 *GF*$^-$.

It cannot be said from this experiment, whether a selection on a certain mutation was done through the fructose.

However, the surviving rates were different for the two mutants:

Table 23: Number of colonies on fructose-containing solid medium

	number of colonies on three plates	average per petri dish
PCC 6803 gtr$^-$	27	9
PCC 6803 GF	4	1.3

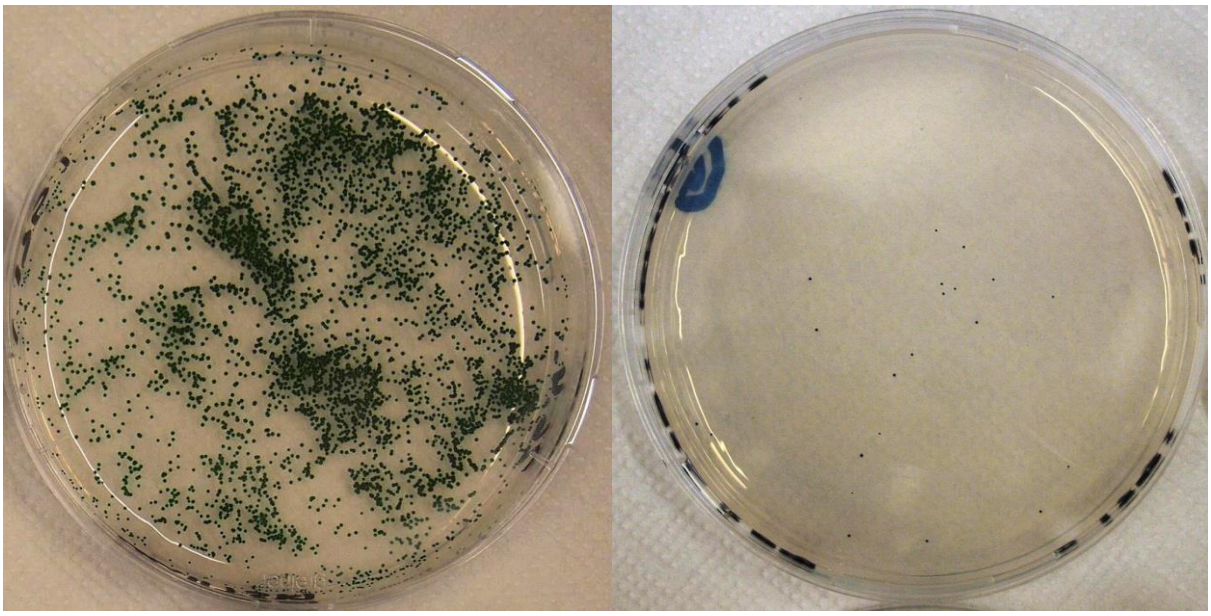


Fig. 40: left: BG11-TS agar plate with PCC 6803 *gtr*$^-$, right: BG11-TS agar plate with PCC 6803 *gtr*$^-$ and 100 mM fructose

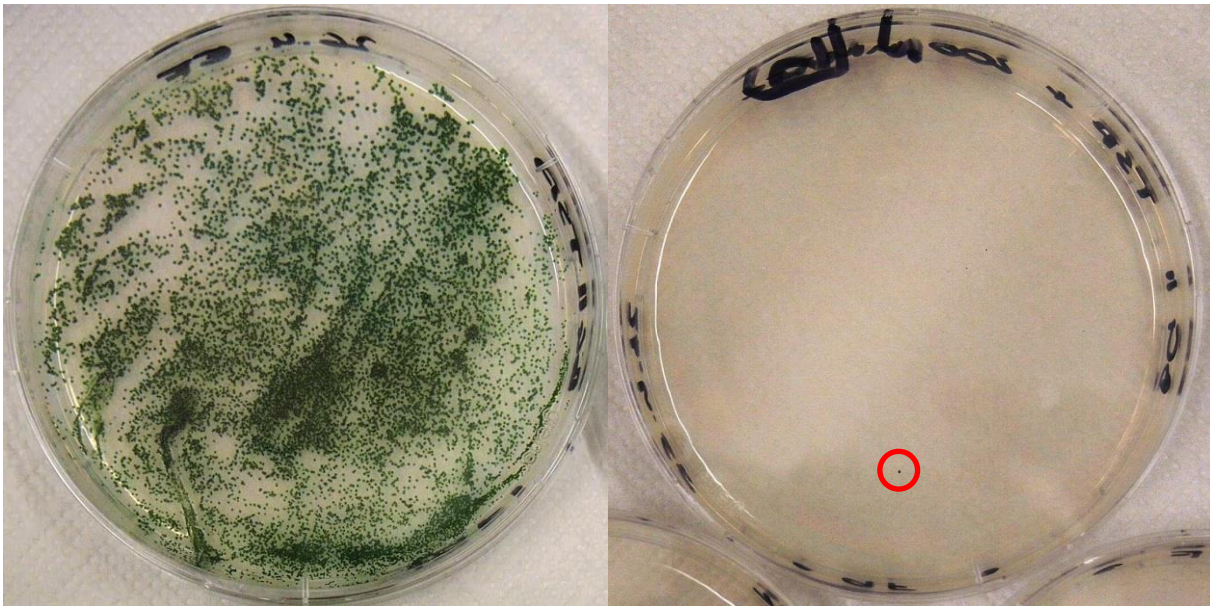


Fig. 41: left: BG11-TS agar plate with PCC 6803 *GF*$^-$; right: BG11-TS agar plate with PCC 6803 *GF*$^-$ and 100 mM fructose

Some of the colonies were used to inoculate 50 mL BG11-TS with and without 100 mM fructose. The cultures were grown under permanent light at 32°C and 60 % air humidity. The cultures with fructose did not grow and the cultures without fructose showed a very weak growth before they died.

PCC 6803 wt, PCC 6803 *gtr*$^{-}$ and PCC 6803 *GF*$^{-}$ under the stereomicroscope

The mutants were examined under a stereomicroscope. There were no obvious morphological differences between the cultures of the mutants and the wildtype.

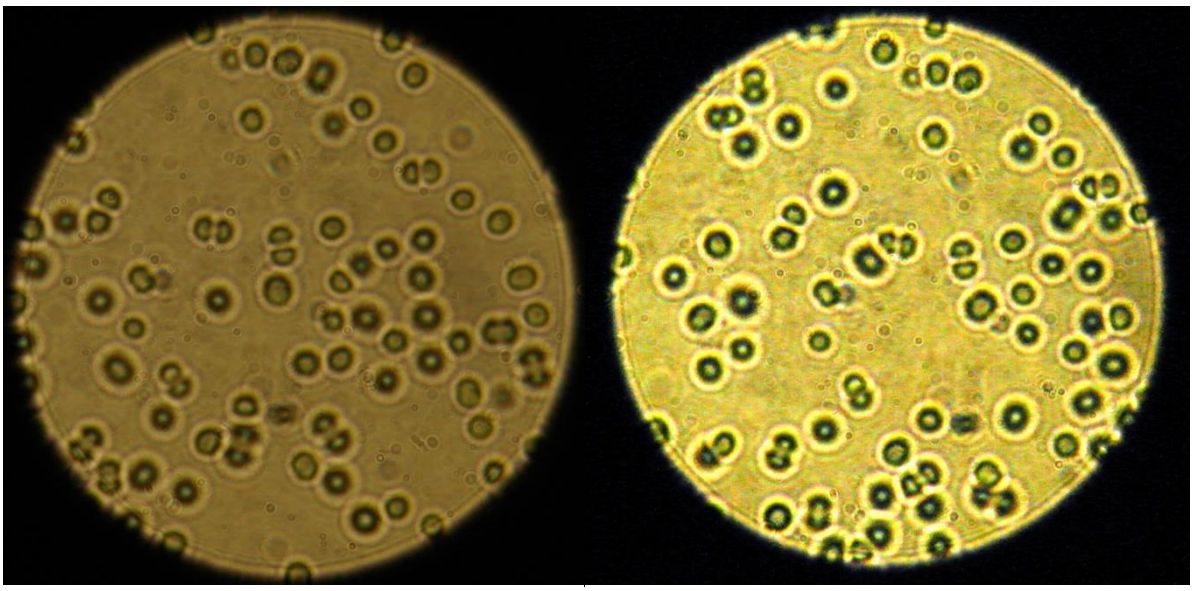


Fig. 42: *Synechocystis* sp. PCC 6803 *GF*$^{-}$ under the stereomicroscope, optical magnification: 1000x

Discussion

Does *alr2514* of *Nostoc* sp. PCC 7120 have a strong secondary structure?

The tough PCR conditions that were necessary for the amplification of the *cox2B* gene and the problems in cloning the gene into the pUC19-vector may indicate a strong secondary structure. It was possible to amplify it under less harsh conditions once it was interrupted by the antibiotic cassette. Nevertheless it was not possible to clone the interrupted gene into the vector pRL278. The reason remains unclear. The gene may build up a secondary structure again once it is cut out.

The results showed that the *sacB* gene on the vector pEFUV3 was not functional. Therefore the ARTO$^-$ mutant of *Nostoc* sp. PCC 7120 could not be obtained.

The mutant *Synechocystis* sp. PCC 6803 *GF*$^-$ has some physiological differences in comparison to the wildtype and the *gtr*$^-$ mutant

In contrary to the wildtype and PCC 6803 *gtr*$^-$,the double mutant PCC 6803 *GF*$^-$ is capable of growing on solid medium without the additional buffer TES-HCl 1 M pH 8.0, 1 (v/v) % in the medium. This could be the result of knocking out the *lacF* gene and marks a significant difference found between PCC 6803 *gtr*$^-$ and PCC 6803 *GF*$^-$.

It was not possible to perform a colony PCR of the mutants. This can be seen as evidence that the membrane and its stability towards temperature has changed in comparison to the wildtype. The *lacF*$^-$ mutant seems to be less sensitive to changes of the pH than the *gtr*$^-$ mutant or the wildtype.

Selection or adaption, which effect does fructose have on the growth of *Synechocystis* sp. PCC 6803?

The experiment on solid medium with high fructose concentrations showed that a selection occurred. Interestingly the rate of colonies per 100 µL bacterial suspension (OD_{730} = 0.1) is much higher for the *gtr*$^-$ single mutant, than for the *GF*$^-$ double mutant. The *GF*$^-$ mutant seems to have more problems adapting to the high sugar concentrations. There are three possible explanations.

The data from the experiment on solid media suggest that fructose is still toxic to the cells. The experiments with radioactively labelled fructose have shown that the sugar can enter the cell. It is not clear yet how the fructose enters the cell or how it is metabolised in

fructose resistant strains of *Synechocystis*. High amounts of fructose may induce spontaneous mutations that allow the growth on fructose. However, in case of one or more additional mutations of the genome a drop of cell density at the beginning of the growth experiment would be expected. The data from the growth experiment in liquid medium showed no decrease of cell density and no significant difference in the first days between the two mutants.

Another possible explanation is the adaptation to osmotic stress. It is known that a high concentration of osmotically active substances can be tolerated by the cell if the concentration is increased slowly (Murata & Suzuki, 2006). The experiment with radioactively labelled fructose showed a higher uptake of radioactivity into cells pretreated with fructose. The cultures that were exposed to high sugar concentrations before and during the experiment had the lowest uptake rate in both mutants. The cultures that were pretreated with low amounts of sugar and then exposed to high amounts during the experiment took up the greatest amount of fructose in both mutants. That could be evidence, that cultures pretreated with low fructose concentrations had time to adapt to the raised osmotic stress.

As can be seen from the results of the experiment with radioactively labelled fructose even low amounts of fructose were sufficient to stimulate the fructose uptake. A further possible explanation for this is that small amounts of external fructose can activate the activity and/or expression of the actual and still unknown fructose carrier in *Synechocystis* sp. PCC 6803 *gtr*$^{-}$ and *GF*$^{-}$ mutants.

The conditions on a solid medium differ from the ones in a liquid medium. Therefore the results of the two experiments ought to be compared with caution. Cyanobacteria grown on solid medium supplemented with fructose did not only grow much slower than the control without fructose, they also seemed to be very sensitive to light.

The liquid cultures that were inoculated with colonies of the solid medium experiments could not be grown in the light chamber as they bleached out at low cell densities or did not grow at all. The colonies that were streaked out onto fresh petri dishes with solid medium supplemented with fructose lead to the same result as the liquid cultures.

The *lacF* gene may change the adaptation to osmotic stress

According to Cyanobase the *lacF* gene is flanked by urea transport system permease proteins on the one side and by a hypothetical protein on the other side. As mentioned in the introduction *slr1202* has similarities to proteins (see table 24) that are involved in the adaptation to osmotic stress in *Synechocystis* (Mikkat & Hagemann, 2000). Mikkat et al., 2000 showed that the gene product of *lacF* has no direct influence on the uptake of osmoprotective substances, as glucoglycerol or trehalose. However, it is not clear whether

slr1202 encodes another osmoactive protein or not. A second gene, *slr1723*, shows high sequence similarities to a second subunit of the glucoglycerol transporter.

Table 15: Protein-blast at Cyanobase, database: Synechocystis sp. PCC 6803

blasted gene	significant alignments	e-Value
slr1202 – permease protein of sugar ABC transporter	*slr0530* – glucosylglycerol transport system permease protein *slr1723* – permease protein of sugar ABC transporter *slr1454* – suphate transport system permease protein	1e-32 5e-04 6e-04
slr1723 – permease protein of sugar ABC transporter	*slr0531* – glucosylglycerol transport system permease protein *slr1454* – sulphate transport system permease protein *slr0530* – glucosylglycerol transport system permease protein	1e-21 6e-07 2e-06

As can be seen in table 24, *slr1202* and *slr1723* have each a high sequence similarity to one subunit of the glucoglycerol transport system. These two genes lie far away from each other on the genome but may encode a single protein.

The results of this work indicate an alteration in the tolerance of osmotic stress caused by a high fructose concentration in the *lacF*$^-$ mutant. On solid medium PCC 6803 *GF*$^-$ could not tolerate fructose as well as PCC 6803 *gtr*$^-$. On the other hand it exhibits an enhanced growth rate in liquid culture.

Fructose can be used for heterotrophic and mixotrophic growth by *Synechocystis* sp. PCC 6803 *gtr*$^-$ and *GF*$^-$

It has been clearly shown that fructose is taken up into the cells by PCC 6803 *gtr*$^-$ and PCC 6803 *GF*$^-$, but the intracellular fate of fructose in these mutants remains unknown. The increased mortality of cultures grown on high fructose concentrations compared to those grown without sugar, as well as the uptake experiments with radioactively labelled fructose may suggest that the carbon metabolism is changed in presence of fructose. The intracellular fate of fructose may depend on the uptake system.

The results of the growth experiment in liquid culture suggest that fructose can be used as carbon source by mutants lacking a functional glucose permease. The monitoring of the optical density showed: the more fructose, the higher the cell number over time.

As can be seen in the results, the cultures exhibit an enhanced growth when the liquid medium is supplemented with fructose. This effect is limited to fructose, as the data showed no enhanced growth for cultures supplemented with glucose. The data therefore suggest

that fructose can be transported via the glucose permease (Flores & Schmetterer, 1986), but that glucose cannot use the transport system of fructose once the gtr-carrier is knocked out.

Growth of *Synechocystis* sp. PCC 6803 *gtr*$^{-}$ and *GF*$^{-}$ mutant on fructose is similar to the fructose adapted growth in the cyanobacterium *Anabaena variabilis* ATCC 29413

The unicellular and non-nitrogen fixing cyanobacterium *Synechocystis* sp. PCC 6803 cannot grow truly heterotrophically in the dark: the cells can use glucose as sole carbon source but a regular exposure to light is necessary for growth (Anderson & McIntosh, 1991). Whether a heterotrophic growth without light activation is possible when *Synechocystis gtr*$^{-}$ mutants are grown on fructose could not be derived from the results of this thesis. But it can clearly be stated that growing the cells on fructose had a profound impact on the culture.

Another cyanobacterium that cannot grow heterotrophically on glucose (Pearce & Carr, 1969) but can do so on fructose is the filamentous and nitrogen fixing cyanobacterium *Anabaena variabilis* ATCC 29413. This strain exhibits different long-term and short-term effects when it is grown on fructose with light and DCMU (photo-heterotrophic growth). For example a threefold higher growth rate in comparison to photo-autotrophically grown cells, a much higher nitrogenase activity, altered chlorophyll content, increased respiration and physiological differences under the microscope (Haury & Spiller, 1981). It has also been shown that the uptake rate in fructose adapted cells was higher than for unadapted cells. The genes involved in the transport of fructose have been identified (Ungerer et al., 2008).

An enhanced growth of fructose grown cells was also proven for the filamentous nitrogen fixing cyanobacterium *Nostoc* sp. PCC 7120 (Stebegg *et al*., 2012). This cyanobacterium does not have the same active fructose uptake system as *Anabaena variabilis* and responds only to high fructose concentrations as the *Synechocystis* strains that were used in this thesis. A BLAST of the protein sequence of *slr1202* showed high similarities to proteins of the two filamentous strains and to the filamentous nitrogen fixing cyanobacterium *Nostoc punctifome*, which has an active fructose uptake system similar to *Anabaena variabilis* ATCC 29133.

Table 165: Protein-blast at Cyanobase, database: *Anabaena variabilis* ATCC 29413 (ava), *Nostoc* sp. PCC 7120 (alr), *Nostoc punctifome* ATCC 29133 (Npun_)

protein sequence	significant alignments	Score (bits)	e-Value	sequence identities	positives
slr1202 (*lacF*)	*ava4638*	368 (944)	e-129	67 %	81 %
	alr0738	367 (942)	e-128	67 %	80 %
	Npun_R6019	355 (910)	e-123	67 %	81 %

The analysis of the amino acid sequence also revealed a high similarity in different single-celled strains like *Syenchococcus elongatus* PCC 7942 (82 %) and others.

The function of *slr1202* remains unclear but the evidences for its role in the metabolism of fructose are increasing. The fact that the growth rate and the rate of incorporated fructose were elevated in PCC 6803 *GF*$^-$ mutants may indicate a regulatory function of the protein.

Synechocystis sp. PCC 6803 *GF*$^-$ may excrete Fe^{III} under osmotic stress

The colour of the supernatant after three to four weeks of growing the *GF*$^-$ mutant on high fructose concentrations may have various origins but one could be the excretion of iron. The iron used by the cell is at oxidation state II and may be oxidised to Fe^{III} by O_2. Iron is redoxactive under physiological conditions and can easily be reduced by an agent as ascorbic acid, as has been done.

The result of the speed vac may also be a hint towards iron. Iron builds complexes with a range of small molecules to larger organic compounds like proteins. This may explain why the experiment with the speed vac did not result in different fractions with different colours.

It has been shown that the exposure of *Synechocystis* sp. PCC 6803 to salt stress leads to an inactivation of the oxygen-evolving machinery and that the cells can be recovered by adding glucose to the medium (Allakhverdiev *et al*., 1999). The uptake of fructose may alter the response to osmotic stress or the recovery of it as well. The iron could also be oxidised by an excreted metabolite of the cell. However, further investigations have to be made to reveal the reason for the specific colour of the supernatant.

melB is essential to *Synechocystis* sp. PCC 6803

The attempt of knocking out the gene *sll1374* did not lead to a homozygous mutant within 12 weeks. This may suggest that this gene is essential for the organism and cannot be completely knocked out. The *melB* gene is annotated by Cyanobase as “probable sugar transporter” and has never been investigated before. The surrounding genes encode hypothetical or unknown proteins.

Zusammenfassung

Cyanobakterien sind die einzigen Bakterien die zur oxygenen Photosynthese befähigt sind. Sie zählen zu den ältesten lebenden Organismen auf dem Planeten und haben maßgeblich zu unserer heutigen Atmosphäre beigetragen. Sie waren vermutlich die ersten Organismen die eine aerobe Atmung entwickelten. Heutzutage dienen Cyanobakterien oft als Modellorganismen.

Die Photosynthese und die Atmung teilen viele Komponenten ihrer Elektrontransferketten. Bei geringem Licht oder im Dunkeln produzieren Respiratorische Terminale Oxidasen (RTOs) Energie indem sie O_2 zu Wasser reduzieren. Drei verschiedene Arten von RTOs sind in Cyanobakterien bekannt. Die Gruppe der Häm Kupfer Oxidasen beinhalten die Cytochrom c Oxidase (Cox), welche die bedeutendste Oxidase für die Atmung darstellt. Diese Gruppe enthält auch die Alternativen Respiratorische Terminale Oxidasen (ARTO). Diese sind sequenzhomolog zur Cox, unterscheidet sich jedoch an wenigen Stellen im aktiven Zentrum in der Subeinheit I und/oder II. Es wird eine Cox-Aktivität für die ARTO vom Typ II vermutet, diese wurde bisher jedoch noch nicht nachgewiesen.

Im Zuge dieser Arbeit wurde ein Selbstmord-Vektor konstruiert mit dem eine ARTO Typ II$^-$ Mutante in dem Cyanobakterium *Nostoc* sp. PCC 7120 erzeugt werden kann. Weiters wurden bereits vorhandene Vektoren und RTO-Mutanten verwendet und verschiedene RTO-Knock-out Varianten herzustellen.

Alle Cyanobakterien können photo-litho-autotroph wachsen. Manche Stämme können sich auch weiterer Wachstumsmodi bedienen, wenn passende organische Wachstumssubstrate vorhanden sind die als Energie-, Elektronen- und/oder Kohlenstoffquellen dienen. Das Cyanobakterium *Synechocystis* sp. PCC 6803 kann heterotroph auf Glukose wachsen, während Fruktose eine antibiotische Wirkung auf diesen Stamm hat. Diese Arbeit hat gezeigt, dass eine Fruktose-resistente Mutante auf hohen Fruktose-Konzentrationen heterotroph wachsen kann und dabei die Wachstumsrate enorm gesteigert wird. Das Monosaccharid gelangt über einen unbekannten Weg in die Zelle in den möglicherweise ein Zuckertransporter involviert ist. Daher wurden zwei vermutliche Zuckertransporter ausgewählt – *lacF* und *melB* – und ausgeknockt. Die Zerstörung des *lacF*-Gens führte zu einer noch größeren Wachstumsrate. Es wurde gezeigt, dass mehr Fruktose in Zellen aufgenommen werden konnte, die zuvor auf Fruktose gezüchtet wurden. Das Genprodukt des *lacF*-Gens hat möglicherweise etwas mit der Regulierung der Fruktoseaufnahme zu tun.

Die *melB*$^-$ Mutante ist nach 12 Wochen noch immer nicht homozygot geworden, was darauf schließen lässt, dass dieses Gen essentiell für den Organismus ist und nicht vollständig ausgeknockt werden kann. Die Funktion dieses Gens ist demnach weiterhin unklar.

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